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MICROBIOLOGICAL, ENVIRONMENTAL AND PROTEOLYTIC ASPECTS IN CHRONIC RHINOSINUSITIS WITH NASAL POLYPOSIS

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Academic Dissertation

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To my family

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LIST OF ABBREVIATIONS

AFRS	Allergic fungal rhinosinusitis
ASA	Aspirin (acetosalicylic acid)
BAL	Bronchoalveolar lavage
CD	Cluster of differentation
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis sine (without) nasal polyposis
CRSwNP	Chronic rhinosinusitis with nasal polyposis
CT	Computed tomography
ECP	Eosinophilic cationic protein
ELISA	Enzyme-linked immunosorbent assay
ESS	Endoscopic sinus surgery
GM-CSF	Granulocyte/macrophage colony-stimulating factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
kDa	Kilodalton
LPS	Lipopolysaccharide
MAP	Mitogen activated protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NAL	Nasal lavage
NGAL	Neutrophil gelatinase associated lipocalin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PMN	Polymorphonuclear cell
RANTES	Regulated on activation, T cell expressed and secreted
RSV	Respiratory syncytial virus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TGF	Transforming growth factor
Th	T helper cell
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-IV.

I. Kostamo K, Richardson M, Virolainen-Julkunen A, Leivo I, Malmberg H, Ylikoski J and Toskala E. Microbiology of chronic hyperplastic sinusitis. Rhinology 2004;42(4):213-8.

II. Kostamo K, Richardson M, Malmberg H, Ylikoski J, Ranta H, and Toskala E. Does the triad of bacteria, fungi and exposure to moisture have an impact on chronic hyperplastic sinusitis? Indoor Air 2005;15(2):112-9.

III. Kostamo K, Sorsa T, Leino M, Tervahartiala T, Alenius H, Richardson M, and Toskala E. In vivo relationship between collagenase-2 and interleukin-8 but not tumour necrosis factor-α in chronic rhinosinusitis with nasal polyposis. Allergy 2005;60(10):1275-9.

IV. Kostamo K, Sorsa T, Tervahartiala T, Richardson M, and Toskala E. The protective role of collagenase-2 (MMP-8) and gelatinase B (MMP-9) in chronic rhinosinusitis with nasal polyposis. (submitted)

ABSTRACT

Chronic rhinosinusitis is one of the most common chronic respiratory tract diseases affecting up to 15% of the adult population in the Western world. It may be perpetuated by factors predisposing to sinus ostial obstruction together with inflammatory changes in the sinus mucosa. The significance of fungal and bacterial infections is doubtful. Chronic rhinosinusitis is regularly associated with asthma, and it is suggested to represent, at least in part, the same disease process. Chronic rhinosinusitis with nasal polyposis (CRSwNP) and asthma share also the characteristic inflammatory features and histopathologic feature of airway remodelling. Remodelling is considered as a key event in the pathogenesis of asthma. It is regarded to be controlled by a delicate balance between the matrix metalloproteinases (MMPs) and their regulators.

The purpose of the present study was to evaluate the microbiological findings, inflammatory features and MMP and tissue inhibitor of metalloproteinases-1 (TIMP-1) expression in CRSwNP. The results were related to the patient history, exposure to moisture and clinical outcome in order to find out possible explanations for the etiology and chronicity of CRSwNP.

Bacterial culture results were similar in patients and in controls and do not explain the chronic course of CRSwNP. The presence of fungi seems to be more common in CRSwNP than chronic rhinosinusitis in general, and they should be actively searched for using microbiological as well as histological methods. Typical outdoor fungal species were found in nasal lavage samples taken from controls in the autumn but not in the winter, reflecting environmental exposure. Exposure to moisture was reported by 46% of the CRSwNP patients, which is in accordance to the Finnish general population. Exposed patients did not differ significantly from non-exposed subjects with regards to microbiological findings, tissue eosinophilia and clinical outcome.

Significantly elevated levels of collagenase-2 (MMP-8) and interleukin (IL)-8 but not tumour necrosis factor-α were found in CRSwNP patients relative to controls. In particular, the activation of mesenchymal-type MMP-8 but not polymorphonuclear-type MMP-8 was associated with elevated IL-8 levels. IL-8 and MMP-8 may form an inductive cytokine-proteinase cascade in CRSwNP pathogenesis and provide a target for novel therapies and an adjunctive diagnostic tool for monitoring CRSwNP treatment. The proteolytic spectrum is different in eosinophilic and non-eosinophilic CRSwNP with the up-regulation of MMP-8 and MMP-9 in non-eosinophilic CRSwNP, suggesting different pathophysiology in

these subgroups. The lack of MMP up-regulation was associated with a poor prognostic factor and worse clinical outcome, representing a possible synergic anti-inflammatory function of MMP-8 and MMP-9 in CRSwNP.

This study provides new information about possible immunologic mechanisms in the pathogenesis of CRSwNP. The recently discovered anti-inflammatory/ defensive properties of MMP-8 and MMP-9 in animal models are reported for the first time in a clinical setting in human inflammatory diseases.

REVIEW OF THE LITERATURE

1. Rhinosinusitis

1.1. Definition and subclasses

The Sinus and Allergy Health Partnership Task Force has defined rhinosinusitis as "a group of disorders characterized by inflammation of the mucosa of the nose and the paranasal sinuses".[23] This definition emphasizes that sinusitis is in general accompanied by concurrent inflammation of the nasal mucosa. Therefore, the term rhinosinusitis is recommended instead of the term sinusitis in both the European Academy of Allergology and Clinical Immunology position paper on rhinosinusitis and nasal polyps and the latest American rhinosinusitis classification.[103,196]

The International Classification of Diseases divides rhinosinusitis into acute and chronic forms according to the duration of the symptoms. Acute rhinosinusitis lasts up to 12 weeks (four weeks in the American Rhinosinusitis Classification) with complete resolution of symptoms, whereas the chronic form persists beyond 12 weeks.[103,196] In immunocompetent persons viral rhinitis usually precedes acute rhinosinusitis and does not require antibiotics for the first seven to ten days unless complicating features are present. If symptoms do not begin to resolve in that time, bacteria are presumed to be involved. So, the current diagnostic clinical criteria for acute bacterial rhinosinusitis, and the indication for antimicrobial therapy as well, include a common cold that is no better after 10 days or worsening disease after 5 to 10 days. The same symptom patterns are accepted also in research definitions for acute bacterial rhinosinusitis.[103,196]

Chronic rhinosinusitis (CRS) is divided into two subclasses: CRS without nasal polyposis (Chronic RhinoSinusitis sine Nasal Polyposis, CRSsNP) and CRS with nasal polyposis (CRSwNP). This subclassification is supported by the fact that CRSsNP differs from the polypoid form in histologic factors, inflammatory profiles and clinical outcome, indicating possible different pathogenic processes involved in these subclasses.[99] In the European position paper CRS is considered as a major finding and nasal polyposis represents a subgroup of this entity.[103] From this definition it follows that nasal polyposis does not exist without concurrent chronic inflammation in the paranasal sinuses. The American consensus conference on rhinosinusitis differs from the European version also in CRS classification. They suggested a classification scheme for CRS based on the presence or absence of three distinguishing features: (1) nasal polyps; (2) eosinophilic or other inflammatory features; and (3) fungal hyphae in sinus mucus.[196] This classification

also classifies possible different causative factors and is mainly intended for research purposes. For clinical use their recommendation was that the minimal classification would be either CRSsNP or CRSwNP.

In addition, other classes are described in the medical literature, such as subacute rhinosinusitis (persisting 4-12 weeks), acute recurrent rhinosinusitis (over four episodes/year with resolution of symptoms between episodes), and acute exacerbation of chronic rhinosinusitis (persistent symptoms between episodes) in recognition of possible differing aetiologies and bacteriology, but the clinical relevance of these subclasses needs to be determined.[175]

1.2. Epidemiology

Rhinosinusitis constitutes one of the most common respiratory tract diseases.[150,281] In a common cold there are frequently abnormalities in the paranasal sinuses.[118,254] In a study of 31 young adults with early common cold these abnormalities were observed using computed tomography (CT) in the maxillary sinus in 87% of the patients, the ethmoid sinus in 65%, the frontal in 32%, and the sphenoid in 39%.[118] In majority of patients the abnormalities resolved within a few weeks without antimicrobial therapy indicating that the common cold is actually viral rhinosinusitis. Moreover, viral infection has been detected in over 80% of patients with common cold and radiological abnormalities in the paranasal sinuses.[254] In immunocompetent patients with the common cold rhinovirus has been the major causative viral agent found in maxillary sinus aspirates, sinus brushing samples or mucosal biopsies in over 50% of patients.[117,242,243,254] Other viruses detected from maxillary sinuses include coronavirus, influenza virus A and B, parainfluenza virus and adenovirus and in children also respiratory syncytial virus (RSV).[143,188,254] Further, RSV infection significantly enhances nontypeable *Haemophilus influenzae* attachment to respiratory epithelial cells, thus providing an example of mechanisms other than ostial obstruction by mucosal swelling by which viral infection results in secondary bacterial infection in acute rhinosinusitis.[143] However, it is estimated that only 0.5-2% of acute viral rhinosinusitis develops into acute bacterial infection.[150]

There are no large epidemiological studies on the prevalence of CRS in Scandinavia or Europe. In a review article an estimated prevalence of 14% in the United States was reported.[150] The prevalence had increased by 50% from 1982 to 1993 in the USA, when estimated by the number of restricted activity days per year, and a similar trend is also seen in the Finnish hospital material.[63,302] In the Canadian cross-sectional study of over 73 000 subjects 12 years of age or older, conducted as a part of the National Population Health Survey, the prevalence of

self-reported CRS was 5%.[56] It was higher in female (5.7%) than in male (3.4%) subjects. The prevalence increased with age, and the sex difference was consistent across age groups. In this study rhinosinusitis were assessed by positive answer to question "Do you have sinusitis diagnosed by a health professional?" Chronicity was defined as "long-term conditions" lasting or being expected to last 6 months or longer. CRS was associated with cigarette smoking, low income, allergy, asthma, and chronic obstructive pulmonary disease. The investigators concluded that the much lower prevalence of CRS among Canadians compared to the Americans may result from geographic differences, as the prevalence of reported rhinosinusitis in the north-eastern region of United States is approximately half that of the southern region.

It is estimated that 20% of patients with chronic rhinosinusitis have also nasal polyposis.[272] This seems a small proportion considering the reported prevalence of nasal polyps in Europe and the current conception of nasal polyposis being a subgroup of CRS. The prevalence of nasal polyposis was reported to be 4.3% in southern Finland in a postal questionnaire survey of a population-based random sample of 4300 subjects.[133] This is in accordance with the 2.11% prevalence of nasal polyposis in the French general population, estimated by a large cross-sectional, case control study using a validated questionnaire.[163] Male predominance is often reported, although this was not seen in the French study, and the prevalence tends to increase with age.[65,163] In the French study allergies were significantly more frequent among nasal polyposis patients. In contrast, in a study of 3000 atopic subjects, the prevalence of nasal polyps was 0.5%, and nasal polyposis is generally considered not to be an allergic condition.[47] Up to over 50% of patients with CRSwNP have associated asthma, which is typically the non-atopic late-onset type and 30% to 40% also have aspirin (ASA) intolerance.[272,281] In children younger than 10 years polyps are rare, and a possibility of cystic fibrosis should be considered in case of a child with nasal polyposis.[281]

1.3. Causative factors

Acute rhinosinusitis is usually an infectious process in which sinus ventilation and drainage are impaired as a consequence of a nasal infection, whereas chronic disease might result from a wide range of processes. Today CRS is acknowledged to be caused by a gradual obstruction due to increased tissue formation in the ostiomeatal complex, leading to impaired sinus ventilation and drainage.[13] As a consequence the oxygen levels inside the sinus decrease, resulting in impaired phagosytosis by decreased opsonization of bacteria, which in turn leads to increased virulence of micro-organisms.[157] Hypoxia can also enhance the production of proteolytic enzymes, which in turn decrease the mucociliary

clearance.[157] These result in enhanced inflammatory and proteolytic reactions together with mucosal edema, and so further obstruct the ostiomeatal complex creating a vicious circle leading to chronic inflammatory disease. Besides the physical pathological mechanism, local and systemic factors predisposing to ostial blockade and infection together with inflammatory changes in the mucosa of the nasal cavity and paranasal sinuses eventually contribute significantly to the chronicity of rhinosinusitis.[13,122]

1.3.1. Anatomical variations

The significance of anatomical variations as a predisposing factor in CRS is controversial. The anatomical variants that may obstruct the ostiomeatal unit include deformities of the uncinate process, pneumatization or paradoxal curvature of the middle turbinate, bulla ethmoidalis, Haller's cells and agger nasi cells.[122] However, the overall prevalence of anatomical variations possibly causing ostiomeatal narrowing has been about 40% both in CRS patients and control subjects.[146] Some studies have reported higher prevalence of specific anatomical variations in CRS patients, for example concha bullosa in 29-33% of CRS patients compared to 11-16% in healthy subjects.[46,146] Also accessory maxillary sinus ostia are found in approximately 30% of patients with CRS and in 10-20% of healthy subjects.[144,146,152] This additional ostium may cause mucus to recirculate from the sinus to the nasal cavity through the natural ostium and back to the sinus through accessory ostium.[152] Recirculating mucus can become recurrently infected. However, there has been no consistent difference in the prevalence of anatomical variations between a symptomatic group and a control group.[146,156]

1.3.2. Microbial factors

The role of viral and bacterial infections in the onset of CRS is not completely clarified.[13,122] The microbiology of CRS differs from that in acute rhinosinusitis. The predominant bacteria in CRS are *Staphylococcus aureus*, coagulase negative staphylococci, *Pseudomonas aeruginosa* and anaerobic bacteria, alone or in combination with facultative aerobic and anaerobic bacteria, whereas *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are recovered from over 75% of patients with acute rhinosinusitis.[102,119,209,326] However, their pathogenicity in CRS is largely unknown. The significance of fungal infections is equally uncertain. Fungal infections may have a more subtle onset and a more chronic course than bacterial rhinosinusitis, and it is estimated that 5-10% of CRS patients actually have fungal rhinosinusitis.[110,190] However, a recent hypothesis that fungal colonization of the nose serves as major inflammatory stimulus in inducing

and sustaining eosinophilic inflammation in most CRS patients needs further studies, as the prevalence of fungal findings, as well as the fungal species recovered, was comparable in the patient and the control samples.[32,45,246] Microbes may be implicated in the pathogenesis of CRS by other mechanisms than those causing persistent infection in the paranasal sinus mucosa, including bone involvement, bacterial biofilms and superantigens.

1.3.2.1. Osteitis

Studies in both animals and humans show that the adjacent bone may become involved in the chronic inflammatory process with changes similar to those seen in chronic osteomyelitis.[158,160,239] Moreover, the inflammation typically spread through the Haversian canals in the bone, resulting in bone changes at a distance from the site of the primary infection.[239] In one study the ethmoid bone specimens, labelled prior to surgery with two short courses of oral tetracyclines two weeks apart, were collected from CRS patients undergoing sinus surgery and from patients operated on for non-inflammatory sinus disease.[158] CRS patients were found to have significantly greater bone remodelling activity, demonstrated by significant separation of the two lines of fluorescence caused by tetracyclines, with histologic changes including new bone formation, fibrosis, and presence of inflammatory cells when compared to controls. The bone turnover in CRS patients was similar to that in osteomyelitis and trauma. Thus, an active inflammatory process occurring in the bone may be a factor in the changes seen in overlying mucosa and in the resistance of CRS to medical therapy.[158,159]

1.3.2.2. Bacterial biofilms

Biofilms, which are communicating organizations of microbes surrounded by glycocalyx, could also explain the resistance of CRS to medical therapy. The vast majority of bacteria exist within a biofilm, including *Pseudomonas aeruginosa*, *Hemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, which are often found in association with otolaryngologic diseases.[248] Bacterial biofilms have been documented on middle ear mucosa and cholesteatoma.[247] There is also evidence of the presence of bacterial biofilms in a rabbit model of *Pseudomonas aeruginosa* rhinosinusitis and in patients infected with *Pseudomonas aeruginosa* who have symptomatic CRS despite medical and surgical treatment.[72,240] Although antibiotic therapy and activated host defence mechanisms are able to kill planktonic cells derived from biofilms and often relieve symptoms, they cannot kill the bacteria in biofilms that constitute the foci of these chronic infections.[70] Mechanical debridement is the only mechanism resolving biofilms,

which may explain the improvement of symptoms of CRS with surgery and irrigation.

1.3.2.3. Superantigens

A number of micro-organisms produce exotoxins (also called enterotoxins) that are able to activate T lymphocytes by cross-linking the MHC (Major Histocompatibility Complex) II on antigen-presenting cells with the variable beta region of the T cell receptor on T cells.[59,168] Both the MHC II molecule binding area and the T cell reseptor binding area for exotoxins are located distant from the antigen-specific binding region.[266] Exotoxins are able to bind to several T cell receptor variable beta region gene motifs. This leads to activation of up to 30% of T lymphocytes, whereas classical antigens can activate less than 0.01% of T lymphocytes, thus the name superantigens.[170] Superantigens can also act as classical antigens and anti-superantigen antibodies are often generated concomitantly. Common microbial superantigens include *Staphylococcus aureus* enterotoxin and some fungi, such as *Alternaria* and *Aspergillus*, may have superantigen activity.[54,266] It is hypothesized that CRS patients, especially those with nasal polyposis, could have microbial superantigen production inside the paranasal sinuses with subsequent sinus mucosal immune activation leading to eosinophilic inflammation in genetically susceptible hosts.[266] In fact, Bachert and colleagues demonstrated specific immunoglobulin (Ig)E to *Staphylococcus aureus* enterotoxins A and B in 50% of altogether 20 patients with eosinophilic nasal polyposis.[12] Moreover, the presence of *Staphylococcus aureus* enterotoxin specific IgE was associated with higher levels of total serum IgE, more severe local disease and increased incidence of systemic manifestations such as asthma, suggesting a possible role of superantigens as disease modifiers**.**

1.3.3. General host factors

1.3.3.1. Allergy

Allergic rhinitis may be a predisposing factor for rhinosinusitis as it may alter the normal physiology of the paranasal sinuses by obstructing the sinus ostia. Although its contribution in CRS is controversial, there is evidence that allergic rhinitis might facilitate development of acute bacterial rhinosinusitis.[6,263] IgE-mediated rhinitis patients have been demonstrated to have more severe paranasal sinus changes in CT scans during a common cold than non-allergic subjects, indicating perhaps a greater risk for development of acute bacterial rhinosinusitis.[6] In a Finnish study of 224 patients with verified acute rhinosinusitis the incidence of allergy, confirmed

by means of an allergy questionnaire, skin testing and nasal smears, was 25%.[263] In addition, probable allergy was found in 6.5% of patients. In the control group the corresponding percentages were 16.5 and 3. However, allergic patients did not differ from non-allergic in the number of prior acute rhinosinusitis.

Several studies have reported an increase in the prevalence of atopic markers and allergy in CRS patients.[125,283,294] Approximately half of the CRS patients have associated allergies, and the prevalence of positive skin prick tests in patients undergoing sinus surgery has been over 80% in some studies.[101,294] Controversially, the prevalence of CRSwNP was only 0.5% in atopic subjects compared to 4.5% prevalence in non-atopic patients.[47,272] Moreover, nasal polyps are statistically more common in nonallergic asthma than in allergic asthma (13% vs. 5%).[272] Furthermore, the total IgE levels and concentration of eosinophilic cationic protein (ECP) and interleukin (IL)-5 in CRSwNS do not differ between atopic and non-atopic subjects, indicating that the systemic allergic phenotype does not correlate with local inflammatory mechanisms leading to eosinophilic inflammation in the sinus mucosa.[11,13,171,172] In CRSsNP the allergic patients have a full T helper cell (Th) type 2 cytokine profile compared to mixed Th1/Th2 cytokine pattern seen in non-allergic patients.[125] Also the degree of eosinophilic infiltration is not markedly different between allergic and non–allergic subjects. Thus it seems that local immunological responses are more important in the development of inflammation in CRS than atopic status.

1.3.3.2. Asthma and aspirin intolerance

CRS is strongly associated with asthma. Approximately 80% of asthmatic patients have allergic rhinitis and 60% have rhinosinusitis.[282] Conversely, up to over 50% of CRSwNP patients have asthma, of whom 30-40% have also ASA intolerance.[272,283] The incidence of rhinosinusitis as identified by radiography in ASA intolerant asthmatics may be over 95% and the frequency of nasal polyps may be as high as 70%.[169] The aspirin-exacerbated respiratory disease is a clinical syndrome defined as a triad of asthma, nasal polyposis and intolerance to ASA and most of the non-steroidal anti-inflammatory drugs.[24] The onset of the disease occurs typically in the early adulthood.[24] The mechanism activating the underlying respiratory disease is unknown, but the pathogenesis of the respiratory reactions in this disease is in part due to altered archidonate metabolism.[305] In Finland Hedman and colleagues examined the prevalence of ASA intolerance and its relation to doctor-diagnosed asthma as a part of a postal questionnaire survey studying the prevalence of asthma in adults.[133] The prevalence of ASA intolerance causing attacks of asthma was 1.2%. It was significantly higher in patients with doctor-diagnosed asthma than without (8.8% versus 0.8%).

1.3.3.3. Immunodeficiency

A global deficiency of all immunoglobulins (congenital or acquired hypogammaglobulinemia) is associated with increased susceptibility to recurrent pyogenic infections, including rhinosinusitis.[327] In addition, IgA deficiency, which is the most common Ig deficiency occurring in 1 of 700 individuals, can lead to recurrent sinopulmonary infections, especially in association with a deficiency in IgG_2, or other IgG subclasses.[229,327] The high incidence of immune dysfunctions was found in a retrospective study of 79 patients with refractory rhinosinusitis referred to the Allergy and Immunology Clinic for immunological evaluation.[55] Abnormalities of T cell function, estimated by in vitro testing of T cell response to recall antigens, alloantigens and T cell mitogens, were the most common defects found in over 50% of 60 patients who underwent the tests. Moreover, 17.9% of patients had low IgG levels, 16.7% had low IgA and 6.2% had low IgM, and common variable immunodeficiency was diagnosed in 9.9%. The high incidence of immune dysfunctions may be explained partly by patient selection, since patients in this study were referral patients from an otorhinolaryngology clinic to an immunology clinic, and thus were already suspected to have possible immune defects. However, similar numbers of immunodeficiency were found in patients with CRS or recurrent rhinosinusitis in a London clinic.[265] Of 74 adult patients, 14 subjects (19%) had low levels of one of the major Ig classes and 23 subjects (31%) had one or more IgG subclass deficiencies. High incidence of Ig deficiency was also found by May and colleagues, who evaluated 245 patients with CRS refractory to prolonged antibiotic treatment.[194] In their study five patients (2.0%) had common variable immunodeficiency and 17 patients (6.9%) had an IgG subclass deficiency. Also acquired immunodeficiency syndrome patients are reported to have an increased occurrence of rhinosinusitis, as well as an increased incidence of features predisposing to rhinosinusitis, such as chronic mucosal thickening and decreased mucociliary clearance.[198,232]

1.3.3.4. Ciliary dysfunction

Decreased mucociliary clearance is considered to be one of the key elements contributing to rhinosinusitis chronicity.[13,122,157,327] Thus diseases affecting ciliary function frequently associate with chronic rhinosinusitis. Primary ciliary dyskinesia is a recessively inherited group of disorders of ciliary structure and/or function resulting in recurrent or chronic respiratory tract infections with mucus retention leading to rhinosinusitis, serous otitis media, rhinitis, and bronchitis.[217] It is a rare disorder with an incidence of 1 in 15000 in the white population. However, in children with recurrent respiratory diseases, primary ciliary dyskinesia can be found in approximately 5%.[52] Kartagener's syndrome, a triad of bronchiectasis,

rhinosinusitis, and situs inversus, is found in 50% of primary ciliary dyskinesia patients.[58] Diagnosis relies on clinical evaluation and electron microscopic analysis of ciliary ultrastructure.[217]

1.3.3.5. Other associated diseases

Another congenital disorder associated with CRSwNP is cystic fibrosis.[178] Nearly every patient with cystic fibrosis has paranasal sinus involvement, but the disease is rare in Finland.[140] However, it should be considered in children and teenagers, who develop nasal polyposis, and also in adult patients, who are operated on for chronic rhinosinusitis when younger than 18 years.[272] Interestingly, in the United States the cystic fibrosis gene mutations have been found to be significantly more common in CRS patients than in controls (7% vs. 2%), even after exclusion of undiagnosed cystic fibrosis patients, suggesting that mutations in genes responsible with cystic fibrosis could promote the development of CRS in the general population.[333] However, the higher prevalence of cystic fibrosis gene mutations was not seen in Finnish CRS patients, reflecting perhaps the low prevalence of these mutations in the Finnish population.[140] Paranasal sinus and nasal cavity involvement is also a common feature in chronic granulomatous disorders, including Wegener's granulomatosis, Churg-Strauss syndrome and rare cases of the paranasal sinus sarcoidosis.[85,113,138]

1.3.4. Other factors

Maxillary rhinosinusitis may also have a dental origin. Periapical abscess or the placement of dental implants in the upper jaw may result in secondary rhinosinusitis with the characteristic features of unilateral disease caused by typical oral pathogens, mainly anaerobes.[36] Also dental obturating material may enter into the sinus and cause chronic infection.[36] Perhaps the most common, and probably most frequently unrecognised, odontogenic cause resulting in radiologic finding consistent with CRS, including sinus mucosal thickening, air-fluid levels in the sinus or complete opacification, is periodontal disease.[1] Sixty % of patients with periodontal disease have been found to have sinus involvement in CT scans compared with 29% of an age-and-sex-matched control population.[1] An even higher incidence of maxillary sinus changes was seen in a small study of 13 patients with advanced periodontal disease.[96] As many as 79% of the patients showed swelling of the mucosa prior to periodontal therapy, compared to only 17% after periodontal therapy.

Secondary rhinosinusitis may also develop due to a foreign body in the nasal cavity mainly in children and mentally handicapped subjects. In adults, nosocomial rhinosinusitis is a complication of critically ill patients needing prolonged intubation.[295] Approximately 25% of patients intubated for 5 days or longer develop rhinosinusitis, and it is more common in patients with nasotracheal tubes than orotracheal tubes.[227] Other risk factors for nosocomial rhinosinusitis are facial trauma, inability to mobilize the patient and prior sinus disease.[295]

Environmental pollution, both outdoor and indoor, may cause irritation of the respiratory mucosa and, in the case of continuous exposure, perhaps also chronic inflammatory disorders of the respiratory tract, including nasal passages. In a German study a weak but consistent statistical association was found between the prevalence of CRS and above average air pollution, estimated by sulphur dioxide, nitrogen oxides and total suspended particles levels.[343] Also ozone is known to cause increased respiratory symptoms and susceptibility to bacterial respiratory infections by inducing inflammatory response and respiratory epithelial damage, including decreases in mucociliary clearance.[215] Oxidant pollutants are able to enhance the generation of proinflammatory cytokines by rhinovirus 16 -infected cells and thus suggest that virus-induced inflammation in upper and lower airways may be exacerbated by concurrent oxidant pollutant exposure.[293] Smoking can cause ciliary loss and cytologic changes in the nasal mucosa, and thus act as a predisposing factor for CRS. In the Third National Health and Nutrition Examination Survey conducted in the United States 1988-1994, direct use of tobacco, but not passive tobacco smoke exposure, was associated with increased prevalence of acute and chronic rhinosinusitis.[186] Smoking is also shown to increase the risk of recurrent disease following sinus surgery and the risk for reoperations.[35,269]

1.4. Lower airway involvement in chronic rhinosinusitis

In recent years there has been a growing understanding of the interactions between upper and lower respiratory tract. The nasal airways, the sinus cavities, and lower airways form a continuous structure lined with ciliated columnar epithelium and share a common embryologic origin. There are also several clinical observations supporting this so called integrated airway syndrome model, which has a wide spectrum of severity varying from rhinitis to asthma and possibly rhinosinusitis.[308,322] Both allergic and non-allergic rhinitis are risk factors for asthma and also for acute exacerbations of asthma.[112,184,273] Epidemiological studies indicate that rhinitis co-exists with asthma in 85% to 94% of patients, compared to approximately 20% in general population.[309,322] Moreover, rhinitis in asthmatic patients is often more severe than in non-asthmatic patients. Several studies have

demonstrated that the severity of asthma correlates with the severity of rhinitis, and treatment of allergic rhinitis with topical corticosteroids or second generation antihistamines has beneficial effects on the outcome of asthma.[66,104,121,273] Furthermore, the lack of symptoms in the lower airways in rhinitis patients or the nasal cavity in asthmatic patients does not mean a lack of involvement. Allergic rhinitis patients with no history of lower airway symptoms have been found to have hyperresponsiveness, inflammation and even tissue remodelling in the lower airways.[89,310] And vice versa, the nasal mucosa of the asthmatic patients shows signs of inflammation even in the absence of nasal symptoms.[280]

Rhinosinusitis is less clearly related to asthma than rhinitis, but the data point to a similar relationship. The paranasal sinus abnormalities in CT examination are found in most patients with moderate to severe asthma, but radiological studies may be misleading since a high percentage of paranasal sinus abnormalities are found in allergic rhinitis also.[34,208] Eosinophilia in the sinus mucosa is stronger in patients with rhinosinusitis and asthma when compared to those with rhinosinusitis alone.[129] Also corresponding histopathologic features of epithelial remodelling, including epithelial erosion and basement membrane thickening, have been seen in sinonasal specimen from chronic rhinosinusitis patients and in asthmatic bronchial mucosal specimens suggesting that CRS and asthma are part of the same disease process.[30,245] Moreover, medical and surgical treatment of CRS have been reported to improve asthma outcomes, estimated by asthma severity, frequency of asthma attacks and need for medication.[43,270,282] However, no firm conclusions can be drawn from these studies with the exception of CRSwNP associated with asthma in aspirin-exacerbated-respiratory disease.[24]

2. Chronic rhinosinusitis with nasal polyposis

2.1. Diagnosis

The lack of pathognomonic symptoms makes objective measurements necessary to accurately diagnose rhinosinusitis. Anterior rhinoscopy is the basic tool to determine pathology existing in the sinonasal passages. During examination attention is paid to possible mucosal edema and/or erythema, polyps or polypoid swelling, crusting, and nasal discharge. Decongestion with topical decongestants is recommended to achieve better visibility to the middle turbinates. Despite the use of decongestants the middle meatus and posterior parts of the nasal cavity stay poorly visualized unless nasal endoscopy is included in the physical examination. Besides research purposes nasal endoscopy during an office evaluation is indicated in patients, who have unilateral disease without septal deviation, who have severe and disabling symptoms, who are immunocompromiced, or after sinus surgery or trauma.[196]

The imaging studies are necessary in assessment of the extent of inflammatory changes within the various sinuses and in defining the anatomy of the sinuses before surgery. It is also indicated if complications are suspected. The modality of choice in sinus imaging is CT, as it gives good resolution of the regional anatomy of the bony structures and mucosa.[103,196] However, the diagnosis of CRS should not be based on CT findings alone, as the prevalence of incidental mucosal changes in an asymptomatic population is approximately 30%, or even higher in the paediatric population.[146,183] Magnetic resonance imaging is superior to CT in differentiating between inflammatory disease and fungal concretions, although rarely used in this indication in Finland, and in establishing the presence of neoplasia. It is also indicated if intracranial complication is suspected.

The research criteria for diagnosis of CRSwNP include: (1) two or more of the following symptoms: anterior and/or posterior mucopurulent drainage; nasal obstruction; facial pressure or pain; and decreased sense of smell persisting beyond 12 weeks, (2) the presence of nasal polyps confirmed in nasal endoscopy; and (3) CT imaging with mucosal changes within ostiomeatal complex and/or sinuses.[103,196] After sinus surgery the presence of polyps is defined as pedunculated lesions on endoscopic examination over six months past operation. For clinical use the requirements for objective documentations are alleviated. Anterior rhinoscopy after nasal decongestation is sufficient to record the absence or presence of polyps. Also sinus CT imaging is optional, although strongly recommended. The differential diagnosis includes antro-choanal polyp; congenital abnormalities, such as

meningocele and meningoencephalocele; benign tumours, such as papilloma and meningeoma; and malignant tumours.[13] These should be suspected especially in case of unilateral disease, but a biopsy of the polyp tissue should always be taken when new nasal polyps are found. Also possible underlying causes, such as aspirin-exacerbated respiratory disease, cystic fibrosis, primary ciliary dyskinesia or paranasal sinus fungal infection, need to be considered in establishing CRSwNP diagnosis.[13,122,272]

2.2. Histology

Histologically CRSwNP differs distinctly from the normal nasal mucosa as well as from CRSsNP. The major histologic hallmark of CRSwNP is eosinophilic inflammation of the mucosa with the exception of nasal polyposis in association with cystic fibrosis and primary ciliary dyskinesia, in which there are lymphocytes in the tissue and neutrophil leucocytes in the secretions.[207,285] The common histomorphologic feature in both types of CRS is thickened basement membrane. CRSsNP is characterized by goblet cell hyperplasia, prominent fibrosis and limited subepithelial edema, whereas in polyp tissue there are areas of squamous cell metaplasia surrounded by normal ciliated columnar epithelia and the stroma is edematous and sometimes fibrotic with a reduced number of goblet cells and submucosal glands.[207] The structure of submucosal glands is also abnormal in CRSwNP, showing signs of cystic degeneration with stagnation of mucus within the distended tubules.[207] The sensory nerves and the autonomic vasomotor and secretory nerves found in normal as well as abnormal nasal mucosa are not seen within the polyp stroma, and the vascularity is sparse.[51] The formation of pseudocysts is characteristic to polyps even in the early stage of polyposis.[11] In pseudocysts there are activated eosinophils and myofibroplasts together with depositions of fibronectin, albumin and probably other plasma proteins in the luminal compartment of the polyps, suggesting a central deposition of plasma proteins as a principle pathogenic mechanism of polyp formation and growth.[11] It is not known why the extravasated plasma is captured inside the stroma and not escaping to the airway surface. The suggested explanations include distance, binding forces, and extracellular matrix damage or abnormality.

There is also evidence of tissue remodelling in nasal polyp tissue. Remodelling is a dynamic process that involves extracellular matrix production and degradation in reaction to different stimuli leading to a normal reconstruction process or a pathologic one. Airway remodelling, together with chronic mucosal inflammation, is a key event in development of asthma involving subepithelial basement membrane thickening, epithelial damage (shedding), smooth muscle hypertrophy, deposition of fibrillar collagen and elastic fibre disruption.[30] Basement membrane

thickening, subepithelial collagen deposition and epithelial shedding have also been demonstrated in CRSwNP.[245,284] The extracellular matrix accumulation has been speculated to be behind the polyp formation and growth in the experimental models.[219] In fact, increased depositions of collagen types III and V, and to a lesser degree collagen I, are found in nasal polyps compared to control nasal turbinate tissue supporting this experimental theory.[204] Fibroblastic cells, including fibroblasts and myofibroblasts, which are the cellular source of extracellular matrix proteins, are abundant in nasal polyps, particularly in the pedicle area, but are not found in the normal nasal mucosa, giving further evidence for the extracellular matrix theory.[332] Moreover, the products of activated eosinophils found in increased numbers in CRSwNP are probably able to stimulate fibroblastic cell mediated collagen deposition.[284,300]

2.3. Inflammatory features

In CRSsNP the inflammatory cells in sinus fluid are predominantly neutrophils, whereas in CRSwNP eosinophils predominate.[13] The presence of eosinophilic inflammation in 263 patients operated on for nasal polyposis and 31 patients operated for non-polypoid CRS was analysed in a retrospective study.[142] All patients with CRSsNP had less than 10% eosinophils with an overall mean of 2%, whereas nearly 90% of samples from CRSwNP patients showed more than 10% of eosinophils and the overall mean was 50%. Similar numbers were found in another study, in which over 70% of CRSwNP patients had eosinophilic inflammation with expression of EG2 (a monoclonal antibody against the secreted form of ECP) activation marker of eosinophils in the majority of eosinophil-positive samples.[300] Allergic and non-allergic CRSwNP patients do not differ in the degree of eosinophilic inflammation.[142] There is also a mild increase in the number of plasma cells and mast cells and evidence of mast cell degranulation in CRSwNP.[154] Mast cells play a key role in IgE-mediated diseases, such as allergic rhinitis, but are also involved in non IgE-mediated inflammatory diseases. Mast cells can express a variety of cytokines, which contribute to eosinophilic inflammation and some are also able to up-regulate chemokines, such as RANTES (Regulated on ActivatioN, T cell Expressed and Secreted), and thus further perpetuate the accumulation of eosinophils. The numbers of macrophages, neutrophils and CD (Cluster of Differentation) 8+ T-lymphocytes are normal, and the number of CD4+ T-lymphocytes may be mildly increased.[196]

Several cytokines and chemokines are reported to be up-regulated in CRSwNP. The cytokine profile is a mixed Th1/Th2 profile with more characteristic Th2 cytokine profile in allergic patients.[123,125] Increased numbers of the Th2-type cytokines IL-3, IL-4, IL-5, IL-13, and granulocyte/macrophage colony stimulating

factor (GM-CSF) and Th1 cytokine interferon (IFN)-γ have been demonstrated in CRSwNP.[11,60,79,126,222] Also pro-inflammatory cytokines tumour necrosis factor (TNF)-α and IL-1β as well as profibrotic cytokines IL-11 and IL-17 are overexpressed in nasal polyps.[11,68,124,204]. In addition, C-X-C-chemokine IL-8 and C-C-chemokines RANTES and eotaxin are overproduced in CRSwNP.[11,31,80] It is not known if IL-8 expression is actually dysregulated in CRSwNP, or does it rather represent the innate immunity response to sinus infection. The latter is supported by the finding of IL-6, IL-8, and IL-11 in both non-polypoid and polypoid CRS, and are thus probably nonspecific mediators of inflammation.[31] Some growth factors, including transforming growth factor (TGF)-α, TGF-β and platelet derived growth factor that most likely induce myofibroblast development and thus contribute to the remodelling process, have been found in polyp tissue.[332] Vascular endothelial growth factor, which is important for inducing angiogenesis and edema, is also increased in nasal polyps and its expression is further upregulated by TGF-β.[69] In addition to cytokines and chemokines, enhanced levels of other inflammatory mediators, such as histamine and tryptase, and immunoglobulins IgA, IgE, IgG and IgM have been found in CRSwNP.[196,235]

2.4. Pathophysiology

2.4.1. Mechanisms

CRSwNP is a multifactorial disease which is associated with miscellaneous diseases including asthma, ASA intolerance, cystic fibrosis and primary ciliary dyskinesia, but affect also otherwise healthy individuals.[13,122,272,281] The inflammatory features of nasal polyps vary in different underlying diseases, thus further pointing to various mechanisms leading to polyp formation.[207,285] The pathophysiological mechanisms of CRSwNP are poorly understood. Several hypotheses have been put forward as the underlying mechanisms of nasal polyposis. The basic problem with all theories is that they cannot explain why polyp formation occurs mainly in one particular area of a few square centimetres in the middle meatus and around the paranasal sinus ostia, although the nasal mucosa is usually universally inflamed. Moreover, these theories do not provide a sufficient explanation of the initiating factor causing the postulated processes. It is as well difficult to distinguish between the cause and consequence of the observed histologic alterations.

The epithelial rupture theory is based on the concept that mucosal oedema, caused by for example allergy or infection, results in rupture of the epithelium.[172] This is followed by a prolapse of the lamina propria through the epithelial defect and

epithelialization of the prolapsed tissue by proliferation and migration of epithelial cells from the surrounding epithelium, resulting in the gradual formation of small polyps. Polyp growth is then further augmented by gravitational effects and/or obstruction of venous drainage in polyp tissue. The vasomotor-imbalance thesis is based on the poorly vascularized stroma of nasal polyps, which lacks a vasoconstrictory innervation.[172] The impaired vascular regulation could lead to increased vascular permeability and reduced detoxification of mast cell products. The prolonged effects of these substances, such as histamine, within the polyp stroma then result in tissue edema. Alterations of the bioelectricity of sodium channels have been found in the nasal mucosa indicating that sodium absorption may be increased in the stroma, which could lead to water retention in the epithelium and the lamina propria of polyps.[26] Moreover, turbulent flow of air in the lateral wall of the nose or pollutant-viral-bacterial-host interactions has been proposed to produce inflammatory changes in the lateral wall of the nose.[221] However, since the increased level of several inflammatory mediators is a prominent feature in nasal polyps, chronic persistent inflammation is undoubtedly a key event in CRSwNP irrespective of the etiology.

2.4.2. Role of eosinophils

Tissue eosinophilia, resulting from increased transendothelial migration and the inhibition of apoptosis of eosinophils, is generally considered to be the main pathogenic event in the development of CRSwNP.[13,129] Eosinophils are able to damage directly the epithelium of the respiratory tract by releasing toxin granule proteins.[172] These granule proteins include ECP, major basic protein, platelet activating factor and eosinophilic peroxidases among others, and can result in ciliary loss, epithelial injury, and nerve damage in the nasal mucosa and thus eventually predispose to chronic inflammation.[76,172] Several pathologic processes probably act in concert by promoting development of eosinophilic inflammation in CRSwNP. The pivotal factor in the pathogenic cascade is considered to be IL-5, since it has several effects on eosinophils.[14,172,339] These effects include differentiation and proliferation factors, chemotactic properties, induction of adhesion molecules increasing recruitment of eosinophils into the tissue, and anti-apoptotic effects.[171,172,279] Furthermore, eosinophils are the only human leukocytes expressing receptors specific for IL-5.[94]

Other processes, which might promote eosinophil accumulation, include local production of IL-3 and GM-CSF, which are able to up-regulate endothelial cell adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, and to cause eosinophil activation and prolonged survival.[123,126,200] Dysregulation of C-C–chemokines RANTES and eotaxin in epithelium and nasal fibroblasts enhance the

local chemotaxis of eosinophils.[123,200] Finally, TNF-α, IL-1 and IL-13 may contribute to the expression of VCAM-1, which selectively mediates the migration of eosinophils and mononuclear cells from the peripheral circulation into the tissues.[172] IL-13 may further enhance IL-5 and eotaxin function.[14,124] Eosinophils in nasal polyp tissue are also able to synthesize IL-3, IL-5, GM-CSF and TNF-α, and thus initiate an autocrine inflammatory mechanism potentially responsible for the persistent inflammation.[14,68,279]

However, the driving force for eosinophil accumulation in CRSwNP is unknown. Moreover, the eosinophil accumulation itself does not explain development of asthma, a disease closely related to CRSwNP, as other clinical conditions with airway eosinophilia, for example eosinophilic pneumonia or sputum eosinophilia in patients with Crohn disease, are not associated with airway hyperresponsiveness and asthma symptoms.[88] For asthma to develop and persist, the airway mucosa must also be susceptible to damage by chronic inflammation. Recent studies suggest that alterations in functions at the epithelial level, with inflammatory cells interacting with epithelial cells, are pivotal in remodelling processes in the pathogenesis of asthma, and a similar relationship may be involved also in CRSwNP.[139]

2.4.3. Dysregulation of epithelium

The microenvironmental theory of lateral nasal airway inflammation is based on increased release of several cytokines by the resident structural cells such as epithelial cells and fibroblasts.[276] These cells are able to produce a number of cytokines, including TNF-α, IL-4, IL-5, IL-6, IL-8, and GM-CSF, which attract inflammatory cells and prolong their survival.[14,207,276,344] In turn these inflammatory cells can produce cytokines such as IL-3, IL-5, TNF-α, and GM-CSF in an autocrine up-regulated fashion and recruit more inflammatory cells.[14,68,279] The damaging effects of eosinophil-derived proteins and mediators on the sinus epithelium may further stimulate the cytokine production. There is also increased expression of intercellular adhesion molecule (ICAM)-1, VCAM-1, and P-selectin in the nasal polyp endothelium and C-C chemokines in the polyp epithelium and submucosal fibroblasts, all of which promote accumulation of eosinophils.[22,124,231,304]

The epithelial functions have been studied extensively in asthma.[57,139] In the bronchial mucosa epithelial cells form a so called epithelial-mesenchymal unit with mesenchymal cells located underneath the basement membrane, such as fibroblasts and myofibroblasts.[57,139] This epithelial-mesenchymal unit controls tissue growth

during development and wound repair as well as inflammation by producing a wide range of proliferative and profibrotic growth factors. Profibrotic mediators directly regulate the mesenchymal cells to produce collagens, reticular and elastic fibres and proteoglycans.[57,92] As the same process probably contributes to the basement membrane thickening and collagen deposition seen in asthmatic bronchial mucosa, it is supposed that dysregulation of epithelial-mesenchymal interactions might be involved in the pathogenesis of asthma. This could lead to impaired injury-repair cycle, blocking the epithelial cells in the "repair phenotype" with continuous release of growth factors and profibrotic mediators.[57] There is also evidence, that Th2 cytokines, especially IL-4 and IL-13, stimulate expression of RANTES, eotaxin, IL-6, ICAM-1 and VCAM-1 in fibroblasts, and thus may influence properties of the epithelial-mesenchymal unit that are relevant to the asthmatic phenotype.[90,139] Taking into consideration the similar inflammatory features and tissue remodelling changes in CRSwNP and asthma together with their clinical co-existence, it is possible that a similar type of dysregulation of epithelium is also present in CRSwNP.

3. Bacteria in rhinosinusitis

3.1. Normal nasal flora

Although the paranasal sinuses are thought to be sterile under normal conditions, the nasal cavity and nasopharynx are colonized with normal flora.[117, 149] The normal nasal flora in adults includes coagulase-negative staphylococci, *Staphylococcus aureus* and *Corynebacterium* species.[147,264] Other aerobic bacteria, including *Streptococcus viridans*, *Neisseria meningitis*, enteric bacteria and *Moraxella* species have been isolated occasionally.[147,264] With the exception of *Staphylococcus aureus*, the potentially pathogenic bacteria are rarely found from the nasal cavities in healthy adults. Controversially, in children also potentially pathogenic bacteria are frequently cultured from the nasal cavity, such as *Streptococcus pneumoniae* in 10-52% of nasal swap samples from pre-school children, *Haemophilus influenzae* in 7-65% and *Moraxella catarrhalis* in 15-58%.[115] Anaerobic bacteria, mainly *Propionibacterium acnes*, *Peptostreptococcus* species and *Bacteroides* species are found in up to 100% of healthy noses.[147,264] In a small study of 12 healthy adults undergoing septoplasty, aerobic bacteria were recovered in sinus aspiration samples from seven and anaerobic bacteria from all twelve subjects, questioning the sterility of sinus cavities.[39] The predominant anaerobic isolates were *Bacteroides* species, anaerobic gram-positive cocci and *Fusobacterium* species and aerobic isolates were beta β-haemolytic streptococci and α-haemolytic streptococci.

The normal nasal flora has a clear impact on the occurrence of infections in other anatomical location and perhaps also in the sinuses. *Staphylococcus aureus* is considered as the most important potentially pathogenic bacteria in the nasal cavity.[107] Approximately 20% of healthy individuals carry *Staphylococcus aureus* consistently in the nose, and an even larger proportion, approximately 60%, has nasal colonization intermittently.[164] Nasal carriage of *Staphylococcus aureus* is a risk factor for the development of wound infections after surgery; infections associated with haemodialysis, peritoneal dialysis; and possibly also bacteremia caused by an intravascular device in the intensive care settings.[164] In the majority of infections the strains isolated from the site of infection have been identical to strains isolated earlier from the carriage site implicating endogenous infection. The significance of nasal carriage of *Staphylococcus aureus* was further demonstrated in a randomized, double-blind, placebo-controlled study of 3864 patients undergoing general, gynecologic, neurologic, or cardiothoracic surgery.[238] The prophylactic intranasal application of mupirocin for 5 days prior to the operation did not significantly reduce the rate of *Staphylococcus aureus* surgical-site infections overall (2.3% in the mupirocin group vs. 2.4% in the placebo group), but

it decreased significantly the rate of *Staphylococcus aureus* infections among the patients who were *Staphylococcus aureus* carriers (4.0% vs. 7.7%, respectively).

Other normal nasal flora bacteria, which may become primary pathogens, include *Neisseria meningitis* and group A streptococci. Meningococci are usually commensal bacteria in humans and only a minority of the nasopharyngeal isolates cause invasive disease.[296] However, in populations living in confined areas such as military recruits, the rates of transmission and carriage are much higher, increasing also a risk of endogenous invasive meningococcal infection. Group A streptococci has been recovered from the throats of 21% of the 136 children who had a clinical and radiographic diagnosis of acute bacterial rhinosinusitis with symptoms lasting for a minimum 10 days and no complaints of sore throat as a prominent condition, whereas the carriage rate in asymptomatic children in the same community during the same season was 5.5%.[328] The authors concluded that a streptococcal infection rather than a viral upper respiratory infection initiated the mucosal inflammation, which led to obstruction of the sinus ostia and a secondary purulent rhinosinusitis.

3.2. Acute rhinosinusitis

It is estimated that only 0.5% to 2.0% of cases of viral rhinosinusitis are complicated by secondary bacterial infection.[150] In general, a sinus infection is caused by a single bacterial isolate in high density but in 25% two bacterial species, both in high density, are recovered.[326] The most important bacterial causes of acute community-acquired rhinosinusitis are *Streptococcus pneumoniae*, *Haemophilus influenzae* and especially in children *Moraxella catarrhalis*.[119,326] In the United States Gwaltney and colleagues summarized the results from several studies from 1975-1990 concerning the microbiology of acute maxillary rhinosinusitis in adults.[119] In maxillary sinus aspirates the most common bacteria were *Streptococcus pneumoniae* and *Haemophilus influenzae*, which accounted for 41% and 35% of the bacterial isolates, respectively. Next in frequency were streptococci other than pneumococci in 7%. In this study *Moraxella catarrhalis* was found in 4% of patients, whereas in studies conducted in children its prevalence has been approximately 15-20%.[326] The microbiology of acute frontal sinusitis is similar to that of acute maxillary rhinosinusitis, whereas in one study evaluating the microbiology of acute sphenoid sinusitis *Streptococcus* species and *Staphylococcus aureus* were the most dominant isolates in equal prevalence.[37,259] Otherwise *Staphylococcus aureus* is an uncommon cause of acute rhinosinusitis both in adults and in children but it may cause serious complications such as intracranial infections and subperiosteal or orbital abscesses.[326] Anaerobic bacteria have been isolated from approximately 5% to 10% of patients with acute rhinosinusitis.[119] Their presence in sinus mucus may indicate primary dental pathology.

The pathogens in nosocomial acute rhinosinusitis differ significantly from those in community acquired disease. Nosocomial rhinosinusitis usually develops in patients requiring extended periods of intensive care involving prolonged endotracheal or nasogastric intubation.[10,227] The etiology is usually polymicrobial with about one-third being Gram-positive organisms, most often *Staphylococcus aureus*, and two thirds Gram-negative, predominating organism being *Pseudomonas aeruginosa*.[295] The other typical pathogens include *Klebsiella pneumoniae*, *Proteus mirabilis, Serratia marcescens,* and *Enterobacter* species and anaerobes, such as *Prevotella* and *Fusobacterum* species.[176,295] Immunocompromised patients with acute rhinosinusitis may also present with atypical causative bacterial agents depending on the type of immunodeficiency and the time of the infection, highlighting the importance of bacterial cultures in the treatment of these patients.

3.3. Chronic rhinosinusitis

The significance of micro-organisms in CRS is less clear. *Staphylococcus aureus*, coagulase-negative staphylococci, gram-negative bacteria and respiratory anaerobes predominate in CRS, but it is difficult to interpret their meaning in the development of CRS.[38,102,326] Coagulase negative staphylococci are recovered in about 40% of patients with CRS, but also in 35% of nasal samples taken endoscopically from the middle meatus in healthy individuals, and probably have no role in the pathogenesis of CRS.[98] Correspondingly, *Staphylococcus aureus* has been reported to occur at similar rates in patients and control subjects.[209] However, *Staphylococcus aureus* grew heavily from patient samples, whereas only light growth was seen in controls. Gram negative rods like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* species, and *Escherichia coli*, which are rarely cultured from samples obtained from healthy individuals or from patients with acute community-acquired rhinosinusitis, are frequently reported in association with CRS.[38,209,326,327] In a study performed in patients with severe CRS, the incidence of gram-negative isolates in samples taken from the middle meatus was 27%, of which over half were *Pseudomonas*.[209] These organisms may be causative, or they might secondarily infect or colonize the altered environment in the sinuses. The latter is supported by the higher incidence of gram-negative rods, especially *Pseudomonas aeruginosa* in surgically treated sinuses than in sinuses not surgically treated, reflecting most likely the change in the sinus microenvironment.

The reported prevalence of anaerobes in sinus aspirates from CRS patients varies from as high as 80-100% in some studies to 0-25% in others, reflecting most likely the technical differences in specimen handling.[122] The predominant anaerobes

isolated in CRS include anaerobic streptococci, *Prevotella* species and *Fusobacterium*.[38,102,326] Recurrences of signs or symptoms of bacterial maxillary rhinosinusitis have been reported to associate twice as often with anaerobes compared to aerobes when bacteria counts were over 10^3 colony forming units/mL, thus implicating their possible role in the disease chronicity.[102] In one study the microbiology of sinus aspirates was studied in patients who failed to respond to antibiotic treatment.[40] Sequential cultures were taken over a period of 34 to 50 days after the initial infection. *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* were the predominant isolates in the initial cultures, but in subsequent cultures these bacteria were accompanied by anaerobes, such as *Fusobacterium*, *Prevotella*, *Porphyromonas* and *Peptostreptococcus*. The initial bacterial infection could have resulted in changes in the sinus microenvironment, such as impaired ventilation, mucous stasis and reduced oxygen level in the sinus, favouring the development of secondary anaerobic infection, which in turn was responsible for the prolonged disease. *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* have also been recovered from CRS patients, some of whom experienced acute sinus symptoms, but in a smaller proportion than in acute rhinosinusitis.[149] It seems likely that these bacterial infections have a role in acute exacerbations of CRS, but it is not clear whether they participate in the basic process initiating chronic sinus disease.

4. Fungal rhinosinusitis

4.1. Presence of fungi in the environment

Fungi are ubiquitous in nature, comprising approximately 25% of the earth's biomass.[53] They have an important role in decomposing organic matter in natural recycling processes. It is estimated, that there are 1.5 million fungus species worldwide, of which 100 000 have been specifically identified.[87] Only about 100 are known to be primary pathogens in humans and animals, causing allergic reactions, infections and ingestion toxicity, and a few hundred more occur as opportunists.[53,87] Most fungi reproduce by forming airborne spores. Fungal spore concentration in the air can easily exceed 100 000 spores/m^3, and thus they constitute the largest portion of bioaerosols in the environment.[53] The most common fungi in both indoor and outdoor settings belong to species *Cladosporium*, *Alternaria*, *Penicillium* and *Aspergillus*, indoor levels being usually 40-80% of outdoor levels.[53] In addition, the common species include *Basiospores* and *Ascospores* outdoors and *Aureobacidium* indoors.[53]

Fungal spore counts in the air show a marked geographic and seasonal variation. The highest counts are most often recorded in the summer and/or autumn months.[2,75] In Finland, the Aerobiological Unit of the University of Turku routinely records the data of air spore counts of selected fungi throughout the year. Their statistics for *Cladosporium* and *Alternaria* show similar seasonal variation (see Figure 1 in the original publication II). The seasonal variation of fungi isolated from the nasal mucus has also been seen in the study of Buzina and colleagues, where they obtained mucus samples by flushing the noses of chronic rhinosinusitis patients and healthy controls.[45] *Cladosporium*, *Alternaria*, *Aureobacidium*, which were the most prevalent fungal species among both groups, showed a significant seasonal fluctuation with a maximum in late summer/early autumn, suggesting merely a reflection of environmental exposure to fungi in the nasal cavity than a cause of the disease itself.

4.2. Fungi in the pathogenesis of chronic rhinosinusitis

In the recent years there has been an increasing interest in the possible fungal aetiology of CRS. Ponikau and colleagues detected fungi in nasal lavage (NAL) samples in 96% of 210 consecutive CRS patients and in 100% of 14 healthy volunteers, when novel mucus collecting and fungal culturing methods were used.[246] An average of 2.7 (2.3 in controls) organisms per patients grew, with a maximum of eight (four in controls) different organisms per subject.

Cladosporium, *Alternaria*, *Penicillium* and *Aspergillus* were the most prevalent genera found in both groups. Of 101 surgically treated patients there were fungal elements present in histological samples in 81% and 93% met the criteria of allergic fungal rhinosinusitis (AFRS). The eosinophils in the mucin occurred independently from IgE-mediated hypersensitivity, and thus the authors proposed a term eosinophilic fungal rhinosinusitis instead of AFRS. In Europe Braun and colleagues reported similar results using similar specimen collection and culture methods as Ponikau and colleagues.[32] There was no marked difference between the number and genera of organisms grown from the patient and the control samples either. The most common indoor fungal species, namely *Cladosporium*, *Alternaria*, *Penicillium* and *Aspergillus*, were the most prevalent fungal species also in this study. Their group conducted another study of 210 CRS patients and 23 healthy volunteers, using the same specimen collection technique as Ponikau, but identification was performed by polymerase chain reaction, with comparable results with the other two studies.[45] Moreover, Catten and colleagues detected fungal DNA in 40% and 42% of nasal swabs taken from CRS patients and controls respectively.[50] Based on these studies it is proposed nearly everyone has fungi in the nose and the mere presence of fungi in the nose and sinuses is not enough to explain their role in the pathogenesis of CRS. Furthermore, the fungal colonization of the nasal and paranasal cavities is present in 15% of newborn babies within the first days of life and increases to 94% in four months, suggesting the ubiquitous existence of fungi in essentially all humans.[174]

The more recent results suggest a much broader role for fungi in CRS patients, such as non-infectious stimuli or targets for the eosinophilic inflammation. Eosinophils are generally regarded to play a role in host defence against larger, non-phagocytosable organisms, such as parasites.[106] Eosinophils also are able to destroy fungi by releasing proteins from granules they contain.[181] In allergic/eosinophilic fungal rhinosinusitis the abundant tissue eosinophils are supposed to be on transit through the mucosa and migrate into the mucus, where they form clusters around fungal elements.[33,246] IgE-mediated allergy to fungi in CRS has been supposed to be behind the eosinophilic inflammation.[267] Even though a number of atopic CRS patients produce specific IgE against fungi, there is no evidence of this IgE production being the actual cause of the disease.[64] The cytokines IL-5, IL-13 and VCAM-1 associated with eosinophil chemotaxis and survival are significantly elevated in sinus tissues from CRS patients independently of their allergy status when compared with healthy controls.[123,124] The *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with *Alternaria* extracts induced production of IL-5 and IL-13 in PBMCs from CRS patients, but not in those from healthy controls.[277] A weaker response occurred after stimulation with *Aspergillus* and *Cladosporium* antigens, but not with *Penicillium*. In addition, PBMCs from CRS patients produced 5.5 times more IFN-γ when stimulated with *Alternaria* extract

than did PBMCs from healthy controls.[277] INF-γ is known to enhance the parasite killing ability of eosinophils.[226] These results suggest that CRS patients seem to respond to common airborne fungi by producing cytokines involved in the eosinophilic inflammation.

4.3. Fungal rhinosinusitis

Although the significance of fungi in the pathogenesis of CRS in general is in dispute, it is known that fungi are capable of invading the paranasal sinuses. It is estimated that approximately 5% to 10% of CRS patients have a diagnosis of fungal rhinosinusitis.[110,190] Fungal rhinosinusitis presents in five distinct clinicopathological forms.[82-84] The invasive forms are acute fulminant, chronic, and granulomatous (also called primary paranasal Aspergillus granuloma) invasive fungal rhinosinusitis. The non-invasive variants of fungal infection are sinus mycetoma (fungus ball) and AFRS, in which fungi are found within the sinus cavity without penetration of the mucosal barrier. The exact diagnosis of fungal rhinosinusitis is difficult to establish because they have many common signs and symptoms and only tissue examination, often with specific fungal stains, provides accurate diagnosis and classification.[82,110] The role of fungal culture in the diagnosis of fungal rhinosinusitis is not determined. Culture is insensitive, as only 20-40% of fungal infections are estimated to be culture positive, but it is often needed for fungal specification.[110] Accurate classification of fungal rhinosinusitis is important, since the treatment and prognosis vary between the disease categories.

4.3.1. Invasive fungal rhinosinusitis

The diagnosis of invasive fungal rhinosinusitis requires findings of rhinosinusitis on radiological imaging and histopathologic evidence of fungal hyphae within sinus mucosa, submucosa, blood vessels, or bone. Classification of invasive fungal rhinosinusitis to three subcategories is based on the type and intensity of the infection estimated by clinical and histopathologic findings.[83] Acute fulminant invasive fungal rhinosinusitis is characterized by rapid spread of the fungus into the adjacent tissues and intracranially often leading to death. Patients are usually immunocompromised having malignant disease, other cause of neutropenia, diabetes, or taking immunosuppressive drugs.[267] They become acutely ill with fever, sinonasal infection, pale and ischemic mucosa, and headache/facial pain, which is in disproportion to physical findings. Anaesthesia of the nasal mucosa and/or facial skin is suggestive of an invasive prosess and does not occur in bacterial infections. Necrotic septal ulcers (eschars) are traditionally described as a hallmark of invasive fungal rhinosinusitis but they are a late finding. Affected

tissues are necrotic, and histopathology show fungal invasion to mucosa and blood vessels, haemorrhage, vasculitis with thrombosis and extensive tissue necrosis with neutrophilic inflammation.[83] The vascular fungal invasion is suggested to be responsible for the fulminant course. The saprophytic fungi of order Murorales, such as *Rhizopus*, *Mucor*, *Rhizomucor* and *Absidia*, and *Aspergillus* species are the most common fungi involved in acute fulminant form.[83] The treatment includes wide surgical debridement, intravenous anti-fungal drugs and correction of the underlying condition. The prognosis depends upon early diagnosis and treatment, and it tends to be poor.

The two other subgroups of invasive fungal rhinosinusitis have more chronic course of the disease, but they as well lead to death if left untreated.[82,83] The chronic invasive fungal rhinosinusitis is clinically less aggressive than the acute fulminant form. It may present as proptosis or orbital apex syndrome, otherwise the clinical features are rather similar to the acute fulminant invasive fungal rhinosinusitis. The histopathologic studies show penetrating fungal hyphae, tissue necrosis with low-grade inflammation. *Aspergillus fumigatus* is the most common offending organism.[83] As in the acute fulminant form, patients are usually immunocompromised. The most common underlying condition is diabetes. Treatment is the same as in acute fulminant fungal rhinosinusitis. However, the infection often recurs and the prognosis is poor.

Granulomatous invasive fungal rhinosinusitis has mostly been reported in Sudan, but also in Pakistan, India and few sporadic cases in the United States.[73,83,199] It is caused by *Aspergillus flavus* in immunocompetent patients. The clinical picture is chronic polypoid rhinosinusitis associated often with proptosis. Typically only one sinus is affected. Histopathology show profuse fungal growth with regional, superficial micro-invasion of fungal hyphae and noncaseating granuloma with giant cells. Eosinophilic inflammation, necrosis and vasculitis are sometimes noted.[319] A concomitant negative mycobacterial stain must be present.[83] Surgical resection followed by oral antimycotic drugs for months is needed.[114] Relapse rate as high as 80% and mortality is reported in patients treated by surgery alone.

4.3.2. Non-invasive fungal rhinosinusitis

The non-invasive forms of fungal rhinosinusitis are more common accounting for over 90% of cases.[110] In sinus mycetoma (fungus ball) there is mucopurulent material within the sinus and histological examination reveals a dense conglomeration of fungal hyphae with a chronic inflammatory response in the adjacent mucosa without tissue necrosis or granulomas and no evidence of fungal

invasion using special fungal stains.[84] Usually only one sinus is affected. Patients present clinically with polypotic chronic rhinosinusitis. Patients are usually immunocompetent, but may have additional underlying risk factors including previous sinus surgery and trauma.[267] The treatment of choice is surgery with the removal of all fungal material and also obstructing hypertrophic sinus mucosa. The prognosis is excellent and usually only one operation is needed.

AFRS is the most controversial form of fungal rhinosinusitis. It was first described in 1983 and termed allergic *Aspergillus* sinusitis because of its histopathologic resemblance to allergic bronchopulmonary aspergillosis.[153] Moreover, it was considered to be caused by a type I immediate hypersensitivity reaction to colonizing fungi leading to allergic inflammation of the sinus mucosa. Since then many other fungal species than *Aspergillus*, mostly dematiaceous fungi such as *Bipolaris*, *Curvularia*, *Alternaria* and *Exserohilum*, have been reported in association with allergic *Aspergillus* sinusitis like conditions and name was changed to AFRS.[268] As also the IgE-mediated mechanism in its pathophysiology is in dispute, the term eosinophilic fungal rhinosinusitis is recommended by some investigators.[246] In AFRS the characteristic feature is allergic mucin, which is a greyish-green material with the consistency of peanut butter containing eosinophils, Charcot-Leyden crystals and sparse non-invasive fungal hyphae. The consensus view of the criteria are lacking, but the latest American rhinosinusitis classification proposed following research criteria: CRS; oedema in the middle meatus or ethmoid area, or nasal polyposis; allergic mucin containing fungal hyphae and no histologic evidence of fungal invasion; CT findings of sinus mucosal disease; and evidence of fungal-specific IgE by means of skin testing or blood testing.[196] The last criterion was excluded from the earlier classification by deShazo and Swain.[86]

The treatment of AFRS is also a matter of controversy. Most reports are from the United States and no international consensus exits. Surgical resection of all mucin and polyps is required.[267] Oral corticosteroids, most commonly prednisone following the modified allergic bronchopulmonary aspergillosis protocol postoperatively for up to one year, has been reported to provide clinical improvement and prolong time to subsequent sinus surgery without significant side effects.[173,268] This is often followed by topical steroids.[173] The role and efficacy of systemic antifungal drugs or antifungal irrigations is also unclear.[99,173] An aggressive allergy management is recommended involving nasal steroid sprays, antihistamines and immunotherapy. In a retrospective analysis of 60 AFRS patients 11.1% of patients receiving immunotherapy were re-operated compared to 33% of patients not receiving immunotherapy, suggesting the potential benefit of immunotherapy in preventing recurrence of AFRS.[21] However, immunotherapy can

worsen patients' symptoms if therapy is started before surgery.[190] Recurrence is common and close follow-up for years is recommended.[173]

5. Moisture exposure

5.1. General aspects

Home dampness, indicated by the presence of water damage, moisture or moulds, is common in Finland and it has been reported in up to 52% of randomly surveyed homes.[167] In the dry Scandinavian climate moisture problems are not caused by general dampness, but rather occur as a consequence of leakage (water damage), capillary movement of soil water, or condensation in cases of insufficient ventilation or inadequate insulation. Adverse health effects associated with moisture exposure have been frequently reported during the last three decades. The possible mechanisms whereby damp building materials may promote diseases include allergic reactions to increased concentrations of mould or mites, toxic non-allergic reactions to products of fungi or other microorganisms, and increased concentrations of other indoor air pollutants associated with moisture exposure.[74] The role of indoor fungi themselves is controversial, since there is no scientific evidence of adverse health effects in occupational settings with extremely high concentrations of fungal spores in the air.[189,258] However, the growth conditions have a major impact on the inflammatory and cytotoxic potential of microbial spores, which could explain the poor correlation between healt effects and the fungal spore count.[258,260]

An association between moisture and mould growth and increased frequency of respiratory, as well as non-respiratory symptoms among the people living or working in a damp building has been demonstrated in several studies.[42,74,167,191,244] Respiratory and mucosal irritation symptoms are the most common but a variety of non-specific symptoms has also been reported in association with moisture exposure, such as joint pain, nausea and vomiting, constipation, fainting spells, backache, headache, fatigue and bad nerves.[191] Exposure to moisture or mould may increase the risk of recurrent respiratory infections including the common cold, bronchitis, rhinitis, or rhinosinusitis.[161,167,241] In addition, a positive association has been reported between moisture exposure and allergic diseases and asthma.[141,161] Moreover, the risk of current asthma in moisture exposure homes was highest among subjects with atopic heredity.

The health data has usually been collected by questionnaires, which rely entirely upon patients' own recollection. This may be problematic in cases of rhinosinusitis because typical symptoms are at best only indicative and the pathological findings in the paranasal sinuses occur also in uncomplicated viral infections, thereby leading to over-interpretation.[13,118] There are also reports of objective findings of

nasal mucosa irritation in association with moisture or mould exposure.[137,218,255,329] An elevation in pro-inflammatory cytokines IL-1, IL-6 and TNF-α in NAL fluid was seen in parallel with respiratory tract and eye irritation in subjects working in moisture-damaged buildings.[137,255] A similar cytokine profile is also seen in viral rhinitis and acute rhinosinusitis, which are characterized by an acute inflammatory cell reaction and no major sequelae for the sinus mucosa.[15] The duration of this up-regulation may be longer when induced by environmental exposure, but only if the exposure continues, and may thus lead to more long-standing mucosal swelling and disturbances in sinus ventilation.[255] The increased concentrations of ECP, a marker of the eosinophil activation, have been detected in NAL samples from people working in damp office building and from subjects exposed to formaldehyde, nitrogen dioxide, and *Aspergillus* species, possibly indicating a link between environmental exposure and chronic inflammation of the nasal mucosa.[218,329]

5.2. Association with chronic rhinosinusitis and fungal rhinosinusitis

There are few studies pointing to the possible effect of the environmental exposure to fungi on CRS and fungal rhinosinusitis. Dennis studied the effect of reduction of the environmental fungal load on the sinus mucosa in 639 patients with apparent CRS.[81] The abnormal sinus mucosa findings persisted in patients who had recurrent exposure to airborne fungi, even after treatment consisting of saline nasal irrigations and antimicrobial nasal sprays containing two structurally different antibiotics, an antifungal and a steroid for 4-10 weeks. The author stated that CRS is likely caused by an immune response to fungal antigen and "As long as fungi remain, so will the irritation and the sinusitis". However, in the absence of a control group of subjects the results of this study should be interpreted with caution. In a Finnish study an association between self-reported rhinosinusitis and elevated serum IgG-levels for ten fungal species, for example, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium cladosporioides* and *Stachybotrus atra*, was reported in teachers exposed to indoor moulds, although there was not a statistical difference in the overall concentrations of 20 mould-specific IgG-antibodies between the study and control groups at the beginning of the study.[234] After school-building remediation the number of teachers reporting episodes of rhinosinusitis decreased from 37% to 11%, but there were no significant changes in IgG concentrations.

Outbreaks of nosocomial invasive fungal infections, usually invasive aspergillosis in the lungs but also some cases in the sinuses, in immunocompromiced patients have been described in association with hospital construction or renovation, contaminated air filtration systems or contaminated construction materials, suggesting that the concentration of fungal spores in the air may play an important

role.[316] In addition, nasopharyngeal colonization may be a risk factor for the acquisition of invasive aspergillosis.[316] However, direct infections caused by indoor moulds are highly uncommon in immunocompetent individuals. In sawmill workers an intense exposure to fungal spores in the working environment did not induce an up-regulation of proinflammatory cytokines in NAL samples.[258] Neither a correlation between mould count and AFRS was seen in a survey done in the United States, although there was a clear geographic distribution of AFRS with the highest incidence in hot and humid areas.[100] In another study the predominant fungal species recovered from air samples from AFRS patients' residences were also isolated from the mucin of its inhabitants.[216] However, the authors were not able to establish an association between sick building syndrome and AFRS, and their results may reflect the influence of environmental exposure to the fungal findings in the nasal cavity rather than the disease pathogens themselves.

6. Matrix metalloproteinases

6.1. Metalloproteinase structure and function

Matrix metalloproteinases (MMPs) comprise a large family of at least 25 different endopeptidases that are collectively capable of degrading almost all components of the extracellular matrix and basement membrane.[193,298] MMPs are part of a much larger metalloproteinase superfamily including also astacins, reprolysins (a disintegrin and metalloproteinases), and serralysins.[28]

All MMPs have a similar domain structure. Every MMP has three conserved motifs assigning proteinases to MMP family, namely a signal peptide, a "pro" region containing the cysteine switch motif PRCGXPD to maintain latency, and a catalytic region that contains the zinc-binding active site.[212,214,233] The active domain binds to three conserved histidines in the sequence HEXXHXXGXXHS.[233] The conserved cysteine residue in the pro domain provides the fourth coordination site for the catalytic $zinc^{2+}$ ion in the inactive state, and disruption of this bond is necessary for enzyme activation. MMPs have additional domains that are important in substrate specificity and recognition or interaction with other proteins or molecules including inhibitor binding.[20,214,233] In the majority of MMPs, these domains are a proline-rich hinge-region and a hemopexin-like COOH terminal. Matrilysins (MMP-7 and MMP-26) lack the hemopexin domain, and in MMP-23 they are substituted by a unique cysteine/proline-rich domain and an IL-1 type II receptor like domain.[116,311] The two gelatinases, MMP-2 and MMP-9, have gelatin-binding fibronectin type II like domains and the membrane-type MMPs contain additional transmembrane-domain anchoring them to the cell surface.[166,330] Along with the domain structure, MMPs also share a similar gene arrangement, suggesting that they eventually have been generated by duplications of an ancestor gene.[233]

The actions of MMPs *in vivo* are complex and diverse. Their main function is presumed to be extracellular matrix degradation in tissue remodelling, as MMPs can cleave extracellular matrix molecules. They have a key role in the embryonic development and tissue morphogenesis including angiogenesis, branching morphogenesis and wound healing.[324] In addition to extracellular matrix remodelling, MMPs influence many cellular functions. They allow cell recruitment and transmigration by cleaving basement membrane components, activating cytoskeletal motor function, modulating cell-surface adhesive molecules and modifying chemoattractants.[297,324] MMPs can alter extracellular micro-environment which results in cell proliferation or apoptosis.[71,145] Furthermore, MMPs modify the activity of biologically active molecules, such as cytokines, defensins, serpins, cell

adhesion and surface molecules and receptors, by direct cleavage, release from bound stores, or modulating activity of their inhibitors.[324] Via these non-destructive functions MMPs are further incorporated in developmental and anti-inflammatory defensive processes.[111,195,228,324] However, MMP knock out mice do not show major development defects as expected by specific MMP functions in vitro.[233,324] Thus, compensatory mechanisms must exist.

Along with physiologic tissue remodelling and repair, MMPs are involved in various pathologic processes, such as inflammation, chronic degenerative diseases, periodontal diseases, cardiovascular diseases and tumour invasion and metastasis.[201,212,214,233,286,323] However, due to the complex nature of MMP involvement and function, it cannot be concluded by their presence alone whether a specific MMP in a pathologic setting is contributing to a reparative or disease process. An example is MMP functions in malignant tumours. The MMPs are frequently overexpressed in various malignancies implicating in tumour growth, invasion and metastasis.[136] Furthermore, they are associated with cell progression to the malignant phenotype, enhanced malignant potential and an adverse prognosis in cancer patients.[5,25,136,299,340] In knockout mice lacking MMP-7 the tumorigenesis, angiogenesis and tumour progression are reduced.[340] Controversially, MMP-8 has been demonstrated to exert anti-tumour activity in the chemical carcinogenesis model of skin tumours as well as *in vitro* in breast tumour cells .[3,18] In addition, the integrin alpha-1 deficient mice have decreased vascularization of tumour xenografts due to increased level of circulating angiostatin generated by increased activity of MMP-7 and MMP-9 in these mice.[251] Thus, increased expression of MMPs may both induce invasiveness of tumours and, paradoxically, lead to production of molecules that limit their growth.

6.2. Regulation of metalloproteinases

Under normal physiological conditions, the activities of MMPs are precisely regulated at the level of transcription, activation of the precursor zymogens, interaction with specific extracellular matrix components, and inhibition by endogenous inhibitors.[212] The importance of each regulatory mechanism varies among different MMPs as well as by clearance and cell types.

6.2.1. Transcriptional regulation

The expression of most MMPs, with the exception of MMP-8 and MMP-9, is regulated transcriptionally.[148] The polymorphonuclear (PMN) cell type MMP-8 and MMP-9 are synthesized in bone marrow, stored in the specific and the secretory

granules, respectively, in the neutrophils, and their activation is mainly regulated by selective subcellular granule release.[338] As a general rule, the MMP genes are not expressed constitutively *in vivo*, and basal MMP levels in cell cultures are low.[148]

A variety of extracellular stimuli may alter MMP gene expression, including cytokines, growth factors, hormones, oncogenic cellular transformations, chemical agents and ultraviolet B irradiation.[136,212] In addition to soluble factors, cell-cell and cell-matrix interactions regulate the MMP gene expression.[212] In general, IL-1, IL-8, TNF-α, prostaglandin E_2, TGF-α, parathyroid hormone and endotoxin up-regulate MMP gene expression, whereas the enhanced expression is down-regulated by suppressive factors including TGF-β, INF-γ, retinoic acids and glucocorticoids.[148,185] Binding of these ligands to their receptors triggers a cascade of intracellular reactions mediated by mitogen-activated protein (MAP) kinases. Activation of MAP kinases culminates in the activation of transcriptional factors, activator protein-1 or polyoma virus enhancer A binding protein-3, in the promoter domain of MMP genes resulting in transcription of the corresponding MMP gene.[136,256]

6.2.2. Activation of metalloproteinases

With the exception of membrane-type MMPs, MMP-11, MMP-23 and MMP-28, which are activated intracellularly by furin, MMPs are secreted as inactive zymogens and are activated by the loss of the propeptide extracellularly or on cell membranes.[236,237] This activation is mediated by proteinases or chemical agents through different pathways with the common features of the disruption of the cysteine residue and zinc ion interaction (cysteine switch), followed by the removal of the propeptide.[315,323].

The three-dimensional structure of MMP pro-domain consists of three α-helixes and connecting loops.[323] The first loop between helix 1 and 2 is a protease-sensitive "bait region". In the substrate-binding cleft in the catalytic domain after helix 3 is an extended peptide region containing the conserved cysteine switch, which forms a fourth ligand of the active-site zinc, keeping the zymogen inactive. The proteolytic activation occurs in a stepwise manner in the bait region. First, the cleavage of exposed bait region partly activates the MMP and probably destabilizes the rest of the propeptide, including the cysteine switch–zinc interaction. This allows intermolecular processing by partially activated MMP intermediates or other active MMPs to occur, resulting in removal of the remaining propeptide and full activation of the MMP.[211] Chemical activation relies also on this cysteine switch

model. The modification of the cysteine switch cause partial activation of the MMP and intramolecular cleavage of the propeptide.[225] As in the proteolytic pathway, full activity is achieved by intermolecular processing.

Several proteolytic enzymes, including plasmin, trypsin, tryptase, neutrophil elastase, cathepsin G, bacterial and fungal proteinases, can cleave off the pro-peptide, resulting in diminished molecular weight and increased catalytic activity.[61,165,203,261,288,289] In addition, recent studies indicate that *in vivo*, the proforms of certain MMPs may also be active while in full-size or in complex with certain proteins.[19,97] Activated MMPs can further participate in processing other MMPs.[206,212]Also various chemical agents, such as organomercurials, gold(I)-compounds, thiol-modifying agents, oxidized glutathione, urea, sodium dodecyl sulfate, chaotropic agents, and reactive oxygens are able to induce proMMP activation.[261,290,291,323] Moreover, low pH and heat treatment can lead to activation.

6.2.3. Inhibition of metalloproteinases

The activity of MMPs can be inhibited by two endogenous inhibitors, including the circulating general inhibitor of MMPs α-2-macroglobulin and tissue-localized tissue inhibitors of metalloproteinases (TIMPs), or by various synthetic inhibitors.

6.2.3.1. Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are the major endogenous MMP inhibitors in the tissues.[323] They are capable of inhibiting the activities of all known MMPs, and thus have a central function in maintaining the balance between extracellular matrix deposition and degradation in different physiological processes.[109] To date, there are four known members in TIMP-family: TIMP-1 to TIMP-4. Although TIMPs have similar inhibitory activities against most members of the MMP family, they differ in many aspects, such as interactions with proMMPs, solubility, regulation of expression, and tissue specific expression.[134] TIMPs are able to control MMP activity in two levels. They inhibit activated enzymes by forming with them tight, noncovalent 1:1 stoichiometric complexes resistant to heat denaturation and proteolytic degradation.[134,289] TIMPs also regulate the activation process by controlling the autocatalytic activation of many proMMPs and forming complexes with proenzymes.[78]

TIMPs also exhibit additional biological functions. TIMP-1 and TIMP-2 have erythroid-potentiating and cell growth–promoting activities along with anti-

apoptotic functions.[105,132,312] In contrast, TIMP-3 has pro-apoptotic activity.[4] TIMP-2, but not TIMP-1, inhibits endothelial cell growth.[205] Moreover, overexpression of TIMP-1, TIMP-2, and TIMP-3 has been found to reduce tumour growth.[323] These activities are distinct from MMP inhibition, and their mechanisms are largely unknown. All these functions of TIMPs can not be reached by using synthetic MMP inhibitors.

6.2.3.2. Synthetic inhibitors

Synthetic inhibitors of MMP fall into three pharmacologic categories: 1) collagen peptidomimetics (batimastat and marimastat) and non-peptidomimetics (BAY 12-9566, AG3340, CGS-27023A and BMS-275291), 2) tetracycline-derivatives, and 3) bisphosphonates.[136] Considering the chronic inflammatory respiratory tract diseases, the most interesting group is tetracycline-derivatives. These agents inhibit both the activity and production of MMPs including membrane-type 1 MMP via multiple mechanisms, including blocking the activity of mature MMPs, interfering with the proteolytic activation of pro-MMP into their active form, reducing the expression of MMPs, and protecting MMPs from proteolytic and oxidative degradation.[108,136] Low-dose (20 mg twice daily for three months) doxycycline therapy in combination with non-surgical periodontal therapy reduced significantly but not completely gingival crevicular fluid and serum MMP-8 levels and improved clinical periodontal parameters over a 12-month period in patients with chronic periodontitis.[95,286,287] The MMP inhibition along with the clinical improvement was detected as well in the murine model of toluene di-isocyanate induced asthma, where administration of doxycycline decreased airway inflammation and airway hyperresponsiveness together with down-regulation of MMP-9 expression.[179] Also macrolide antibiotics, which may be as effective as prednisolone in chronic rhinosinusitis in long-term, low-dose administration, has been found to exert suppressive activity on MMP-2 and MMP-9 production from nasal polyp fibroblasts *in vitro*.[151,331] Thus, macrolide antibiotics probably represent another group of antibiotics already in the clinical use demonstrating anti-MMP activity as a part of their therapeutic profile.

6.3. Classification

MMPs are classified into six main subfamilies, according to their substrate specificity, primary structures, and cellular localization: the matrilysins, collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs. The last subfamily compromises of seven MMPs, which can not be classified in the

above categories. Here three different type MMPs studied in the CRS patients are discussed in detail.

6.3.1. Matrilysin (MMP-7)

MMP-7 together with MMP-26 belongs to the matrilysins subfamily of MMPs. They lack the C-terminal hemopexin domain as a common structural feature and are therefore considerably smaller than MMPs in general. MMP-7 is produced by blood monocytes, macrophages and epithelial cells in exocrine glands, intestine and airways, whereas expression of MMP-26 is found in placental macrophages and stromal cells.[44,77,93,301] MMP-7 is constitutively expressed in non-injured, normal epithelium, implying its role in tissue homeostasis. MMP-7 can proteolytically activate α-defensins, an important component of mucosal innate immunity, in mouse small intestine and it may serve a similar function also in the mucosal epithelium of the respiratory tract.[233,341] Moreover, significantly increased (25-50 fold) expression and activation of MMP-7 were found after bacterial exposure in mucosal epithelial tissues, including airways.[187] This up-regulation was specific for mucosal epithelial cells, and the expressions of other MMP examined (MMP-1, MMP-2, MMP-9, MT1-MMP/MMP-14) were not influenced. MMP-7 is also markedly up-regulated in migrating tracheal epithelial cells after injury, whereas MMP-7 knock-out mice showed no evidence of epithelial migration resulting in severe wound-repair defect.[93] These results demonstrated that catalytic activity of MMP-7 is needed to facilitate cell migration and is essential for the repair of respiratory mucosal epithelium.

6.3.2. Collagenase-2 (MMP-8)

MMP-8 is a member of an interstitial collagenase subfamily of MMPs, including also collagenase-1 (MMP-1) and collagenase-3 (MMP-13). Collagenases have a unique capacity to degrade native fibrillar type I, II and III collagens, major structural components of the extracellular matrix. These interstitial collagens are composed of three polypeptide chains arranged in a rigid triple helix conformation, rendering them resistant to degradation by proteinases other than the interstitial collagenases. Collagenases can also degrade type VII, VIII, X collagens, gelatin and proteoglycans.[130] Furthermore, they can inactivate serpins, including α1-antitrypsin and α1-antichymotrypsin, and also MMP inhibitor α2-macroglobulin.[197,292,338] MMP-8 is the most effective collagenase to initiate type I collagen degradation and it is an initiator of collagen breakdown at the sites of inflammation, as well as in the atherosclerotic plaques and aortic aneurysms.[135,342] MMP-8 is also implicated in the pathogenesis of several chronic inflammatory

diseases characterized by excessive influx and activation of PMN cells, such as cystic fibrosis, rheumatoid arthritis, periodontal disease, and chronic skin wounds.[192,220,250,286] MMP-8 seems to have an anti-tumour activity in breast carcinomas and skin tumours and anti-inflammatory properties in mouse model of asthma and allergen-induced airway inflammation.[3,18,111,228]

MMP-8 was earlier regarded solely as a PMN specific MMP and designated as a neutrophil collagenase.[130] However, the expression of the less glycosylated mesenchymal MMP-8 have been detected in various non-PMN cells, including synovial fibroblasts and endothelial cells, chondrocytes, gingival sulcular epithelial cells, odontoplasts and plasma cells.[62,127,230,307,325] Mesenchymal MMP-8 is also expressed by benign and malign tumourgenic epithelial cell lines *in vitro* and by tumour cells in squamous cell carcinoma of the head and neck region *in vivo*.[17,202] In addition, inflamed human bronchial epithelial cells express MMP-8 mRNA and protein *in vivo*.[253] The highly glycosylated PMN-type MMP-8 is stored in the 75-80 kilodalton (kDa) latent form in the specific granules in the neutrophils and converted to a 65-70 kDa active form upon selective degranulation. After release from granules, a significant part of MMP-8 is associated to the membrane of neutrophils and exerts pericellular proteolysis.[228] In non-PMN cells the inductive *de novo* synthesis of mesenchymal type MMP-8 is transcriptionally regulated.[62,127,202,230] The 55-60 kDa latent mesenchymal type isoform is converted to 45-50 kDa active isoform upon activation by chymotrypsin, cathepsin G, trypsin-2, MMP-3, MMP-7, MMP-10, and reactive oxygen metabolites.[203,210]

6.3.3. Gelatinase B (MMP-9)

Gelatinase A (MMP-2) and gelatinase B (MMP-9) comprise the gelatinases subfamily of MMPs. MMP-2 and MMP-9 have three gelatin-binding fibronectin type II –like domains that are important for their activity on type IV collagen, gelatin, and elastin.[278] Type IV collagen, the principal substrate, is the main component of basement membranes and thus, MMP-2 and MMP-9 are usually expressed by endothelial cells, although other cells, such as stromal fibroblasts, macrophages and tumour cells, can also express them.[9,275] Gelatinase expression also correlates with the invasive potential of various tumours.[299] Gelatinases control the transepithelial migration of inflammatory cells (T cells, monocytes, eosinophils, and mast cells) by controlling the formation of transepithelial C-C-chemokine gradients in which chemokines are strongly expressed on the apical surface of epithelial cells relative to the interstitium.[67] When inflammatory cells are recruited to the lungs, the recently extravasated cells traverse the pulmonary interstitium and the airway epithelium to enter the airway lumen, where they are cleared. Lack of MMP-2, affecting only eotaxin (CCL11), and MMP-9, affecting eotaxin, CCL7,

and CCL17, disrupts the normal cell trafficking into the airway lumen and favouring their accumulation in parenchyma. Thus, by generating several transepithelial chemokine gradients, MMP-9 is essentially implicated in the resolution of allergic inflammation.

MMP-9 is secreted from degranulating neutrophils as a glycosylated 92 kDa latent form, and converted to a 82 kDa form upon activation via proteinase cascade by trypsin-2, neutrophil elastase, active MMP-2, MMP-3 and MMP-7.[9,289,338] Moreover, MMP-9 can be expressed and released in a 120 kDa complex form with 29 kDa neutrophil gelatinase associated lipocalin (NGAL).[162] MMP-9 is also produced by many cells that accumulate in allergic airway inflammation, such as eosinophils, mast cells, natural killer cells, dendritic cells and alveolar macrophages.[9,48,193,223] In acute IgG immune complex induced lung injury the MMP-9-deficient mice had less severe lung injury when compared to wild-type controls, suggesting that MMP-9 is involved in the pathogenesis of the lung injury.[334] In contrast, MMP-9 expression was up-regulated *in vitro* in the respiratory epithelial cells cultured from human nasal polyp tissue or human bronchial tissue during the wound repair.[180] A marked MMP-9 activation occurred only in cells involved in the repair process located at the leading edge of a wound. Moreover, MMP-9 expression paralleled cell migration speed, implicating its role in the physiologic repair process in the respiratory epithelium.

6.4. Metalloproteinase expression in chronic inflammatory airway diseases

Tissue remodelling is a characteristic feature in many chronic lung diseases, such as loss of alveolar walls in emphysema, subepithelial fibrosis in asthma, intra-alveolar fibrosis in idiopathic pulmonary fibrosis, and bronchiectasis in cystic fibrosis, involving extracellular matrix production and degradation.[9,193] Since MMPs constitute the major proteolytic enzyme system responsible for the extracellular matrix remodelling, they have been investigated especially in lower respiratory tract diseases. Considering the fact that MMPs also have a central, non-destructive, role in key biological activities in inflammation, innate immune response, and infection, MMPs are highly likely implicated in several ways in chronic inflammatory airway diseases too.[306,324] Failures in these non-destructive processes, leading to sustained inflammation, have been emphasised of being caused by excessive extracellular protease activity. However, an excess of TIMP over MMP has been proposed to favour airway remodelling in asthma.[57,193] Moreover, recent studies on MMP knock-out mice have shown that MMP deficiency can promote airway inflammation.[111,195,228]

MMP-9 has been found to be the predominant MMP in blood, sputum, bronchoalveolar lavage (BAL) fluid, and bronchial mucosa biopsy in asthmatic patients.[9] TIMP-1, a major endogenous inhibitor of MMP-9, is also elevated in stable asthma to molar concentrations exceeding those of MMP-9.[193] TIMP-1 is considered to have fibrogenic properties resulting from inhibition of MMP-9 and promotion of growth of fibroblasts and myofibroblasts.[131] The TIMP-1/MMP-9 imbalance could lead to pathologic collagen deposition leading to thickened airways with restricted airflow. High TIMP-1/MMP-1 molar ratio correlates with the disease severity and predicts also poor response to oral corticosteroids in asthmatics, supporting the fibrogenic theory.[29,193,321] In contrast, an increase in MMP-9, resulting in a low TIMP-1:MMP-9 ratio, has been demonstrated in decompensated asthma, under acute asthma exacerbations or after allergen challenge, suggesting that MMPs may be involved in the different tissue degradation processes and/or cellular functions in different states of the disease.[182]

Significant increases of MMP-1, MMP-2 and MMP-8 have also been reported in the sputum or BAL fluid from asthmatics.[49,252,303] MMP-8 is of special interest in the lung remodelling, as it hydrolyzes most efficiently type I collagen, which is also the major interstitial collagen type in human lung extracellular matrix.[130] The secreted MMP-8 in BAL fluid has been detected to convert into active form in steroid naïve or uncontrolled severe asthma, but not in clinically stable disease, nor in healthy controls.[252] In severe disease both PMN-type and mesenchymal-type MMP-8 isoforms were observed to be converted to active forms. Moreover, a focal and strong MMP-8 immunoreactivity was found in bronchial epithelial cells especially in the injured areas, indicating perhaps more advanced destructive and irreversible tissue injury rather than inflammation itself. High amounts of functionally active collagenases, mainly MMP-8, have been shown in BAL fluid from patients with bronchiectasis, a disease characterized by irreversible tissue injury.[253,271] Like in asthma, the MMP-8 expression correlated with the disease severity.[252,253,271] However, the functions and mechanisms of action of MMP-8 are not well established in inflammatory disorders.

Recently, MMPs have been shown to exhibit unexpected anti-inflammatory properties in respiratory diseases. Owen and colleagues found in lipopolysaccharide (LPS)-induced lung inflammation greater accumulation of PMN cells and increased myeloperoxidase activity in the alveolar space in MMP-8 knock-out mice when compared to wild type mice, representing defensive role of MMP-8.[228] This anti-inflammatory function of MMP-8 was demonstrated and extended also in allergen-induced airway inflammation, in which MMP-8 knock-out mice were found to have enhanced neutrophilic inflammation in BAL fluid and eosinophilic infiltration in airway walls together with increased levels of IgE and

IgG1 in serum and of IL-4 in BAL fluid.[111] In addition, MMP-9 knock-out mice have been found to have significantly heightened airway inflammation after allergen challenge including increased number of eosinophils in BAL fluid and lung tissue accompanied by enhanced levels of IL-4, IL-5 and IL-13 and pro-eosinophilic chemokine eotaxin in comparison with wild type mice.[195] Moreover, inflammation persisted longer in MMP-9 knock-out mice. Similar inflammatory features, namely enhanced levels of Th2 cytokines and eosinophils, were found both MMP-8 and MMP-9 knock-out mice, suggesting a synergic function of MMP-8 and MMP-9 in the control of airway allergic inflammation.

6.5. Metalloproteinase expression in chronic rhinosinusitis

There is only limited data concerning MMPs in CRS. However, CRS, especially the polypoid form, and asthma share several similar inflammatory features, suggesting a common pathophysiology.[30,245,283] The elevated levels of MMP-9 and TIMP-1 were found in CRS, whereas in nasal polyposis MMP-7 and MMP-9, but not TIMP-1, were up-regulated.[177,336] Immunohistochemical staining revealed the accumulation of MMP-7 and MMP-9 in the subepithelial region and around blood vessels in the nasal polyps.[336] In particular in the nasal polyposis the inflammatory cells stained positive for MMP-7 and MMP-9, but not for TIMP-1, were found inside the pseudocysts, pointing to their degradative function in nasal polyposis pathogenesis. In addition, increased MMP-9 inside the extracellular matrix after sinus surgery was found to predict poor healing quality, although it did not correlate with edema, fibrosis, or overall inflammatory reaction.[337] The investigators did not study MMP-9/TIMP-1 molar ratio, which has been evaluated in predicting corticosteroid efficacy in asthma. Lechapt-Zalcman and colleagues studied MMP-2 also in nasal polyposis patients, but the level of both latent and activated forms did not differ from those observed in control samples.[177]

There are three studies done by Suzaki and his workgroup in Japan examining anti-MMP-effect of pharmaceutical agents in nasal polyposis or rhinitis *in vitro*.[8,151,213] Every study evaluated the influence of the drug in question on the MMP-2 and MMP-9 levels and MMP-2 and MMP-9 mRNA expression in cell cultures after stimulation of MMP expression by TNF-α. Both fluticasone propionate and roxitromycin caused significant suppression of MMP levels and MMP mRNA expression in nasal polyp fibroblasts.[151,213] In the third study fexofenadine hydrochloride inhibited as well the MMP levels and MMP mRNA expression in nasal polyp fibroblasts taken from patients with allergic rhinitis and in nasal mucosal cells from septal deformity patients without allergy.[8] The investigators concluded that inhibitory action on MMP activity and tissue remodelling may underlie the clinical efficacy of these drugs in nasal polyposis and allergic diseases.

The interpretation must be done with caution, as the MMP functions in the upper respiratory tract diseases are largely unknown. Moreover, in the perennial allergic rhinitis patients TIMP-1 and TIMP-2 mRNA were present in large amounts in the nasal mucosal samples, whereas only minimal quantity of MMP-1, MMP-2, MMP-3 and MMP-9 mRNA were found in the same samples.[274] There was no significant difference in the MMP and TIMP levels between patients and healthy controls, raising the question about the significance of MMPs in the allergic rhinitis.

AIMS OF THE STUDY

I. To study the full clinical picture, including patient history, microbiology and histology, of chronic rhinosinusitis with nasal polyposis in patients operated on for this condition in order to find out possible causative factors for the chronicity of the disease.

II. To investigate the impact of moisture exposure on chronic rhinosinusitis with nasal polyposis and to evaluate the seasonal variation of fungal and bacterial findings in the healthy nose.

III. To evaluate possible up-regulation and activation of MMP-8 in chronic rhinosinusitis with nasal polyposis, and to study the relationship between MMP-8 and pro-inflammatory cytokines IL-8 and TNF-α.

IV. To study up-regulation and activation of metalloproteinases MMP-7, MMP-8 and MMP-9 in relation to TIMP-1 in chronic rhinosinusitis with nasal polyposis. The MMP function, protective or destructive, is further evaluated by comparing patients with more active disease, estimated by tissue eosinophilia and a need for re-operations during three-year period.

MATERIALS AND METHODS

1. Study population

Study population consisted of 30 patients undergoing a paranasal sinus operation due to chronic rhinosinusitis with nasal polyposis (Table 1). All patients had a history of chronic rhinosinusitis lasting longer than twelve weeks in spite of appropriate conservative treatment. They also had findings of mucosal swelling and retention in some of the paranasal sinuses in preoperative CT-scan. The presence of polyps was assessed by anterior rhinoscopy and further confirmed during the endoscopic operation.

Study I. All 30 patients were enrolled to Study I. Twelve patients were men (mean age 47.3 y, range 36-68 y) and 18 patients were women (mean age 48.4 y, range 28-66 y). Nineteen patients (63%) had earlier had one or more sinus operation including polypectomy, antrostomy, endoscopic sinus surgery (ESS) or a Caldwell-Luc-operation. Moreover, two patients had had a (rhino)septoplasty. Fifteen patients (50%) reported allergic rhinitis, 17 patients (57%) had asthma and five of them (29%) had ASA intolerence. These five patients were all operated on at least once previously. Altogether four patients (13%) were immunocompromised: three subjects had specific IgG subclass deficiency and one had IgG III and IgM deficiencies. All four patients had had several operations.

Study II. Twenty-eight patients (11 males, mean age 45.9 y, range 36-68 y; 17 females, mean age 47.9 y, range 28-68 y) were enrolled to the second study. Nineteen of them (68%) had earlier had one or more nasal or sinus operation. Fifteen patients (54%) had self-reported allergic rhinitis, 17 patients (61%) had asthma and five of them (29%) had ASA intolerance.

Study III. Thirteen patients (five males, mean age 44.6 y, range 36-53 y; eight females, mean age 47.4 y, range 28-68 y) were recruited to the third study. Nine patients (69%) had been operated on earlier, eight patients (62%) had self-reported allergic rhinitis, eight patients (62%) had asthma and two of them (25%) had also ASA intolerance. All patients used topical corticosteroids.

Study IV. Twenty-four patients (11 males, mean age 45.9 y, range 36-68 y; 13 females, mean age 46.5 y, range 28-68 y) were enrolled. Thirteen patients (54%) had allergic rhinitis, 14 patients (58%) had asthma, and three asthmatic patients (21%) were ASA intolerant. All except one patient had used topical corticosteroids,

Table 1. The history of moisture problems, patient history, previous nose or sinus operations, results for tissue eosinophilia and fungal examinations, and clinical diagnosis in 30 patients operated for chronic rhinosinusitis with nasal polyposis (CRSwNP)

Patient age (years)		Exposure to moisture	Patient history	Previous nose or sinus operations	Result for: Tissue eosinophilia	Fungal staining of sinus mucus	Fungal hyphae in tissue specimen	Culture	Clinical diagnosis
Male	36	-	A, AR, ID	+	+	Yeast cells	-	-	AFRS-like sdr
Female	49	-	AR	+	-	Fungal hyphae	+	-	Mycetoma
Male	36	H	-	+	+	-	+	-	AFRS-like sdr
Female	53	H	A, AR	+	+	-	-	-	CRSwNP
Female	47	-	A, AR	-	+	-	-	-	CRSwNP
Female	29	-	-	-	-	-	-	-	CRSwNP
Female	53	H	A, ASA	+	+	-	-	-	CRSwNP
Male	44	-	-	-	+	-	-	-	CRSwNP
Male	68	-	A	-	+	-	-	-	CRSwNP
Female	53	-	A	+	+	-	-	-	CRSwNP
Male	58	W	-	-	-	Fungal hyphae	+	-	Mycetoma
Female	44	-	AR	+	+	-	-	-	CRSwNP
Male	40	H	A, AR, ID	+	+	-	-	-	CRSwNP
Female	44	W	AR	-	Not taken	-	Not taken	-	CRSwNP
Female	56	-	ID	+	-	-	-	-	CRSwNP
Female	38	-	AR	-	-	-	-	-	CRSwNP
Female	28	-	A, AR	-	-	-	-	-	CRSwNP
Female	52	W	A, AR	+	-	-	-	-	CRSwNP
Female	66	-	A, ASA, AR	+	-	-	-	-	CRSwNP
Female	56	H	A, ASA	+	+	-	+	-	AFRS-like sdr
Female	42	-	A, AR	+	-	-	-	-	CRSwNP
Male	36	-	A, ID	+	+	-	-	-	CRSwNP
Female	54	W	A, AR	+	+	-	-	-	CRSwNP
Male	48	W+H	A, AR	+	+	-	-	-	CRSwNP
Male	53	-	AR	+	+	Fungal hyphae	+	*Aspergillus niger*	AFRS-like sdr
Male	45	W	A	+	+	-	-	-	CRSwNP
Male	41	W	-	-	-	-	-	-	CRSwNP
Female	51	W	A, ASA	+	+	-	-	-	CRSwNP
Male	62	NA	-	-	+	-	-	-	CRSwNP
Female	56	NA	-	+	+	Fungal hyphae	-	*Aspergillus fumigatus*	Mycetoma

H, Moisture problem at home; W, Moisture problem at work; NA, Not ascertained; A, Asthma; AR, Allergic rhinitis; ASA, Aspirin intolerance; ID, Immunodeficiency; AFRS-like sdr, Allergic fungal rhinosinusitis like syndrome

two patients had short course of oral prednisone preoperatively, and two patients had low-dose, long-term oral prednisone. During the three-year period following the initial operation nine patients (38%) were re-operated, of whom eight patients had tissue eosinophilia.

Twenty healthy volunteers (nine males, mean age 37.4 y, range 30-46 y; 11 females, mean age 39.9 y, range 29-60 y) from the Department of Otorhinolaryngology without any history of recurrent or chronic rhinosinusitis or nasal or sinus operations served as volunteer controls.

2. Patient interview

At the time of the operation the patients were interviewed about their medical history, which included questions on allergic manifestations, some background factors and environmental factors. The questions were derived from the

Miljömedicin 040 questionnaire. The questions on allergy were as follows: “Have you now or have you ever had asthma; hay fever or other allergic rhinitis?” The asthmatic patients were queried about ASA intolerance. Moreover, the subjects’ former nasal or sinus operations were noted. Moisture problems at home or at work were assessed by positive answers to at least one of the following questions: (1) “Has there been an odour of mould at your home or workplace in the last three years?” (2) “Have you had visible mould growth or damp stains at your home or workplace in the last three years?” (3) “Has there been repair due to the moisture damage at your home or workplace in the last three years?”

3. Specimen collection

Patients’ microbiological (Study I and II) and metalloproteinase (Study III and IV) specimens were collected during the endoscopic operation. Mucus from the paranasal sinus was placed into an empty sample tube for fungal studies and in modified Stuart transport medium (Transpocult; Orion Diagnostica, Espoo, Finland) for bacterial culture. A specimen for fungal staining was taken with a sterile cotton swab and placed directly onto a microscope slide. The slide was then air-dried. Mucus from the affected paranasal sinus was placed into a sample tube containing 1 mL phosphate buffered saline (PBS) and frozen directly at -20°C for metalloproteinase analysis. The biopsy for histological analysis was obtained from the same paranasal sinus as the mucus.

NAL method, described earlier by Hirvonen and colleagues, was used with some modifications for collecting samples from the control subjects and also for cytokine analysis from 15 patients.[137] In patients NAL was done preoperatively to prevent a possible impact of operation on cytokine levels. In brief, each nostril was flushed through with 5 mL of PBS using a 5 mL sterile syringe and a sterile butterfly cannula of about 3 cm in length. The cut end of the cannula was placed inside the nostril posterior to the nasal vestibulum. The patient closed the nostrils by pinching them firmly together and leaned forward. PBS was pushed back and forth twice and finally collected into the syringe. Any residual PBS remaining in the nostril was collected in a pan placed underneath the nose and collected into the syringe.

About 2 mL of NAL fluid was placed into an empty sample tube for fungal examinations. Bacterial culture was collected by dipping a sterile cotton swab soaked in activated charcoal in the fluid. It was placed into a Transpocult tube. 1 mL of NAL fluid was separated for MMP and TIMP-1 analysis and frozen at -20°C. The NAL sample was further processed for cytokine analysis. The sample was centrifuged, the cells were re-suspended in about 2 mL of supernatant and the

suspension was incubated for 24 h at 37°C. The suspension was centrifuged and the supernatant was collected and frozen at -70°C for later cytokine analysis.

To study the seasonal variation in controls the NAL was done twice from the same subjects: first in January and again in September.

4. Specimen handling

4.1. Bacterial culture

The standard methods used in the diagnostic laboratories of Helsinki University Central Hospital to culture and identify both bacteria and fungi were used. The bacterial samples were inoculated onto the following media: chocolate agar for the isolation of aerobes; blood agar with colistin to select for *Streptococcus* sp; fastidious anaerobic agar for all anaerobes; blood agar with neomycin and vancomycin for *Bacteroides* sp; and thioglycollate broth for enrichment culture. Aerobic cultures were incubated at 36°C in an atmosphere containing 5% CO_2. The plates were examined after 24 h and 48 h. The anaerobic cultures were incubated in an atmosphere of 4-10% CO_2 (the GasPak, Becton Dickinson Microbiology Systems, Cockeysville, Md) and examined after 48 h. Both aerobic and anaerobic plates were cultured five more days and examined at day seven in case of negative growth after 48 h.

4.2. Fungal staining and culture

The fungal specimen was first vortexed to mechanically disperse the mucus. Calcofluor white fluorescence staining was carried out using the processed sample or the sample placed directly onto a microscope slide and the preparation was examined using a fluorescence microscope with 40-fold magnification. The remaining sample was plated out on Sabouraud dextrose agar containing penicillin 6 mg/L and streptomycin 26mg/L. The cultures were incubated at 28°C and 37°C and examined at day seven and day ten.

4.3. Eosinophil staining and histology

For eosinophil staining the lavage sample was centrifuged 1500 revolutions per minute for 10 minutes. Cells were fixed on a microscopy slide air-drying. The slides were stained first in eosin-solution (Merck), rinsed quickly in water followed

by rinsing with ethanol and stained again in methylthionin-solution (Merck). The slides were finally rinsed with water and ethanol and air-dried. The sample was considered as positive, if any eosinophils were detected under the microscope.

All histological samples were stained with hematoxylin and eosin and with Periodic acid-Schiff staining. If the Periodic acid-Schiff staining was negative for fungi the Gomori methenamine silver staining method was done.

4.4. Immunofluorometric assay for MMP-8

MMP-8 levels were analyzed by a time-resolved immunofluorescence assay as described by Hanemaaijer and colleagues.[127] The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica Oy Ab, Kauniainen, Finland) were used as a catching and tracer antibody, respectively. Europium-chelate was used to label the tracer antibody. The samples were diluted in assay buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/L diethylene-triamine-penta-acetic acid and incubated for one hour, followed by incubation for one hour with the tracer antibody. Enhancement solution was added, and after five minutes, the fluorescence was measured using 1234 Delfia Research Fluoremeter (Wallac, Turku, Finland). The levels of MMP-8 were expressed as ng/mL and converted to pmol/mL for the study IV.

4.5. Enzyme-linked immunosorbent assays for MMP-7, MMP-9 and TIMP-1

MMP-7, MMP-9 and TIMP-1 concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Biotrak ELISA systems (Amersham Biosciences UK Ltd, Buckinghamshire, UK) were used for MMP-7 and -9 according to the manufacturer's protocol and DuoSet ELISA development Systems (R&D Systems, Minneapolis, USA) for TIMP-1, correspondingly. All samples were analysed in duplicate. The so called secondary antibody in each kit was conjugated with horseradish peroxidase and tetra methyl benzidine was used as a substrate. The absorbance was measured at 450 nm using Labsystems Multiskan RC (Thermo Bioanalysis Corporation, Santa FE, USA). The levels of MMPs and TIMP-1 were expressed as ng/mL and converted to pmol/mL.

4.6. Western immunoblotting for MMP-8, MMP-9 and TIMP-1

The molecular weight forms of MMP-8, MMP-9 and TIMP-1were analysed by Western immunoblotting analysis using specific rabbit polyclonal anti-human MMP-8, MMP-9 and TIMP-1 antibodies as described by Prikk and colleagues.[252] After sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) run under non-reducing conditions, the proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmont, California). After the unoccupied sites were blocked with 3% gelatin, the membrane first reacted with the primary antibody (1:500) and then with the alkaline phosphatase conjugated secondary antibody. The molecular weight forms of MMP-8 were visualized to photographic film using enhancement chemiluminescence systems (Amersham Biosciences). Quantization was carried out with a Bio-Rad Model GS-700 Imaging Densitometer using analysis program of Bio-Rad [253]. Data is expressed as densitometric arbitrary units. Recombinant human proMMP-9 and TIMP-1 were used as positive controls for MMP-9 and TIMP-1. Human neutrophil and rheumatoid synovial culture media were used as positive controls for PMN-type and mesenchymal type MMP-8 isoforms, respectively.

4.7. Gelatin zymography for MMP-9

Gelatinolytic activity was analysed by zymography using 1mg/mL gelatine as substrate. After SDS-PAGE electrophoresis the gels were washed in 50mM Tris-HCl, 2.5% Tween 80 and 0.02% (w/v) NaN_3 for 30 min; followed by the same buffer supplemented with 1 μM $ZnCl_2$ and 5 mM $CaCl_2$ for 30 min; and finally incubated in 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% (w/v) NaN_3 at 37°C for 24 h. The reaction was interrupted by staining the gels with 0.1% Coomassie Brilliant Blue R250 and then destained in 10% acetic acid, methanol solution. The gelatinolytic activity was visualized as clear bands against a blue background and the band intensity was quantified with a Bio-Rad Model GS-700 Imaging Densitometer using analysis program of Bio-Rad. Pre-stained low range molecular weight SDS-PAGE standards were used as molecular weight markers.[289]

4.8. Analysis of cytokines

The concentrations of IL-8 and TNF-α in the supernatant of NAL fluid were measured using the Quantikine human ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacture's protocol. Cytokine levels were expressed as pg/mL.

5. Statistical analysis

The studies I and II were mainly descriptive and no statistical analysis was done.

In the studies III and IV data are expressed as median and 25-75 interquartile range (IQR). The MMPs and TIMP-1 levels in the CRS groups and in the control subjects were compared using the Mann-Whitney U test. The exact probability test was used to compare the need for re-operation between eosinophilic and non-eosinophilic CRSwNP patients (study IV). Correlations were described with correlation coefficient *r* and their respective *P*-value. *P*-values under 0.05 were considered significant.

6. Ethics

The study was approved by the Ethical Committee of Helsinki University Central Hospital. Written consent was obtained from each patient.

RESULTS

1. Microbiology of chronic rhinosinusitis with nasal polyposis (I, II, IV)

1.1. Bacterial findings (I, II)

Table 2 lists the bacteria found in patients and in controls. Twenty-eight out of 30 patients (93%) had positive bacterial culture. In ten cultures (33%) two or more bacteria were cultured. At least one species of aerobic bacteria was cultured from 27 patients (90%) and anaerobic bacteria from three patients (10%). The most common aerobic bacteria was *Staphylococcus aureus*, which was isolated from ten patients (33%); followed by coagulase negative Staphylococci (23%). The only anaerobic species found was *Propionibacterium acnes*. In the control group 14 cultures (70%) were positive in January and in September bacteria were isolated from all samples. The two most common bacterial species in both control group samples were coagulase negative Staphylococci (40% in January vs. 68% in September) and *Staphylococcus aureus* (21% vs. 11%, respectively). The only anaerobic bacterium found in the control samples was *Propionibacterium acnes* in one subject in September.

Table 2. Bacteria isolated in sinus mucus samples from the chronic rhinosinusitis with nasal polyposis patients and from nasal lavage samples collected in the winter and in the autumn from healthy controls

	Patients n (%)	**Controls n (%)**	
Bacteria	**n=30**	**Winter n=20**	**Autumn n=19**
Staphylococcus aureus	10 (33%)	5 (25%)	2 (11%)
Coagulase negative *Staphylococci**	7 (23%)	8 (40%)	13 (68%)
Propionibacterium acnes	3 (10%)	-	1 (5%)
Streptococcus viridans	2 (7%)	1 (5%)	-
Stenotrophomonas maltophilia	2 (7%)	-	-
Escherichia coli	2 (7%)	1 (5%)	2** (11%)
Klebsiella pneumoniae	2 (7%)	1 (5%)	-
Klebsiella oxytoca	2 (7%)	1 (5%)	-
Haemophilus influenzae	2 (7%)	-	-
Proteus mirabilis	2 (7%)	-	-
Streptococcus pneumoniae	1 (3%)	-	1 (5%)
Corynebacterium sp.	1 (3%)	-	1 (5%)
Neisseria meningitidis	-	-	1 (5%)
Enterobacter sp		-	1 (5%)
Culture positive	28 (93%)	14 (70%)	19 (100%)
Two or more bacteria	10 (30%)	2 (10%)	6 (32%)

* includes *Staphylococcus epidermidis*

** Includes one unspesified coliform rod

1.2. Fungal findings (I, II)

Seven out of 30 patients (23%) had one or more findings suggesting fungal infection (Table 1). Three patients had clinical and histological findings consistent with sinus mycetoma. The other four patients were considered as having AFRS-like syndrome. No invasive fungal infections were discovered. Fungal staining of the mucus was positive in five patients: one patient had yeast cells and four patients had fungal hyphae. The cultures were positive in only two patients: one *Aspergillus fumigatus* and one *Aspergillus niger*. Both patients had also fungal hyphae present in the sinus mucus sample. There were five specimens positive for fungal hyphae in histological samples stained with Periodic acid-Schiff. Only in two of these five specimens were fungal hyphae visible in samples stained with hematoxylin-eosin staining. Gomori staining yielded no additional positive findings.

In the control group all NAL samples were negative for fungi in January, but in September fungi were isolated from NAL samples of three subjects. Both *Cladosporium* and *Alternaria* were cultured from two subjects and *Cladosporium* from one. No seasonal effect on fungal findings was seen in the patient group.

1.3. Eosinophils (I, IV)

Tissue eosinophilia was present in 19 histological specimens (63%) (Table 1). Secretion eosinophilia was also examined from 24 patients. Of these 24 samples, eight were positive for eosinophils and four of them had also tissue eosinophilia. All of these eight patients had asthma or allergic rhinitis. Of seven patients with some finding of fungi none had secretion eosinophilia, but five had tissue eosinophilia. In the control group all samples were negative for eosinophils.

Sixteen out of 24 patients (67%) entering the Study IV had eosinophilic inflammation determined by eosinophils present in tissue biopsy obtained from the same paranasal sinus as the mucus and stained with hematoxylin and eosin.

2. Chronic rhinosinusitis with nasal polyposis and moisture exposure (II)

Thirteen of 28 patients (46%) reported a certain degree of moisture damage at work or at home within the last three years (Table 1). Seven patients (54%) reported moisture problems at work, five patients (38%) at home and one patient (8%) both at work and at home. Two patients reported an odour suggestive of mould at work

and occupational symptoms also in fellow workers. The remaining 11 patients had visible mould, water damage or repair done due to the moisture damage at home or at work. The level of indoor exposure in the control subjects was not ascertained.

When the 13 patients who had reported moisture damage were compared with those 15 patients who did not, the occurrence of one or more positive fungal findings was 23% vs. 20%, positive bacterial culture 92% vs. 93%, two or more bacterial species in the culture 23% vs. 33%, and tissue eosinophilia 75% vs. 53% respectively. Seventy-seven % vs. 60% of the patients previously had undergone one or more nasal or sinus operations. The variants of fungal rhinosinusitis distributed evenly: one mycetoma patient and two with an AFRS-like syndrome in both groups. The bacterial species isolated from patients with or without a history of moisture problem did not differ from species isolated from healthy controls.

3. MMPs, TIMP-1 and cytokines in chronic rhinosinusitis with nasal polyposis (III, IV)

3.1. Results for MMP, TIMP-1 and cytokine analysis (III, IV)

Study III. The concentration of MMP-8 in CRSwNP patients' samples (median 83.0 ng/mL, IQR 24.8-570.2 ng/mL) was significantly elevated in relation to the control samples (median 3.6 ng/mL, IQR 1.1-21.3 ng/mL) ($P<0.01$). In addition, the concentration of IL-8 was significantly increased in patients (median 419.9 pg/mL, IQR 60.0-689.8 pg/mL) when compared to controls (median 25.2 pg/mL, IQR 9.2-61.7 pg/mL) ($P<0.01$). There was also a statistically significant difference in the median concentration of TNF-α in CRSwNP patients (median 0.8 pg/mL, IQR 0.6-1.8 pg/mL) in relation to controls (median 0.1 pg/mL, IQR 0-0.3 pg/mL) ($P<0.01$), but the concentrations were overall very low.

When eight patients with asthma were compared with five non-asthmatic patients, the median concentrations of MMP-8 were 31.1 ng/mL and 188.6 ng/mL, IL-8 were 583.9 pg/mL and 79.7 pg/mL, and TNF-α 0.8 pg/mL and 0.6 pg/mL, respectively. The differences were not statistically significant.

Study IV. The concentrations of MMPs and TIMP-1 together with their molar ratios are shown in Table 3 and 4. The eight patients without tissue eosinophilia had significantly increased levels of MMP-8 and MMP-9, together with the enhanced MMP-8/TIMP-1 and MMP-9/TMP-1 molar ratio, when compared to 16

Table 3. The median and 25-75 interquartile range of MMP and TIMP-1 concentrations (pmol/mL) in controls; in CRSwNP patients with tissue eosinophilia (Eos+) and without it (Eos-); and in same patients re-operated on during 3-year-period after initial operation (Re-op+) and not re-operated on (Re-op-)

	MMP-7	MMP-8	MMP-9	TIMP-1
Controls (n=19)	0.002 (0-0.007)	0.84 (0.22-3.95)	0.09 (0.07-0.24)	0.54 (0.31-0.74)
Patients Eos+ (n=16)	0.007 (0-0.010)	0.53 (0.35-6.18)#	0.15 (0.08-0.80)#	1.26 (0.92-1.45)*
Patients Eos- (n=8)	0.011 (0.008-0.015)*	23.17 (2.25-68.07)*	0.74 (0.35-2.84)*	0.54 (0.19-0.87)
Patients Re-op+ (n=9)	0.005 (0-0.009)#	0.38 (0.04-2.58)#	0.09 (0.06-0.15)#	0.98 (0.38-1.27)
Patients Re-op- (n=15)	0.010 (0.007-0.017)*	5.31 (1.21-36.80)*	0.69 (0.31-3.21)*	1.19 (0.72-1.45)*

* p<0.05 in comparison to controls

# p<0.05 Eos+ in comparison to Eos- and Re-op+ to Re-op-

Table 4. The median and 25-75 interquartile range of MMP/TIMP-1 molar ratio in controls; in CRSwNP patients with tissue eosinophilia (Eos+) and without it (Eos-); and in same patients re-operated on during 3-year-period after initial operation (Re-op+) and not re-operated on (Re-op-)

	MMP-7/TIMP-1	MMP-8/TIMP-1	MMP-9/TIMP-1
Controls (n=19)	0.002 (0-0.018)	1.56 (0.80-7.46)	0.22 (0.10-0.67)
Patients Eos+ (n=16)	0.006 (0-0.009)	0.73 (0.29-5.60)#	0.38 (0.11-1.12)#
Patients Eos- (n=8)	0.020 (0.012-0.1)	54.89 (20.83-75.41)*	2.97 (1.83-4.26)*
Patients Re-op+ (n=9)	0.004 (0-0.006)	0.45 (0.26-6.76)#	0.12 (0.09-0.70)#
Patients Re-op- (n=15)	0.010 (0.005-0.046)	16.12 (0.93-54.89)	1.50 (0.62-3.49)*

* p<0.05 in comparison to controls

# p<0.05 Eos+ in comparison to Eos- and Re-op+ to Re-op-

eosinophil-positive CRSwNP patients and 19 controls, whereas in the eosinophil-positive patients these parameters were at the same level compared to controls (Table 3 and 4). Furthermore, there was a statistically significant difference in the median concentration of MMP-7, but not in MMP-7/TIMP-1 ratio, in eosinophil-negative patients compared to controls. However, the MMP-7 concentrations were overall very low (IQR 0.008-0.015 vs. 0-0.007 pmol/mL).

Nine patients were re-operated on during the three-year period after the initial operation. Eight of these nine patients had tissue eosinophilia, but there was no statistically significant difference in risk for re-operation between eosinophilic and non-eosinophilic CRSwNP patients (P=0.17). However, in nine re-operated patients the levels of MMP-7, MMP-8 and MMP-9 together with the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were significantly lower when compared with 15 patients who did not need re-operation (Table 3 and 4). Among eosinophil-positive CRSwNP patients the eight re-operated patients, in relation to eight non re-operated subjects, had lower MMP-8 (median 0.38 pmol/mL, IQR 0.03-2.63 pmol/mL vs. median 2.17 pmol/mL, IQR 0.47-9.86 pmol/mL), MMP-9 (median 0.07 pmol/mL, IQR 0.06-0.14 pmol/mL vs. median 0.91 pmol/mL, IQR

0.31-2.26 pmol/mL), MMP-8/TIMP-1 ratio (median 0.36, IQR 0.20-3.65 vs. median 1.86, IQR 0.37-6.73) and MMP-9/TIMP-1 ratio (median 0.11, IQR 0.08-0.33 vs. 0.88, IQR 0.43-1.96). The differences in MMP-9 concentration and MMP-9/TIMP-1 ratio were significant ($P<0.01$ and $P<0.05$, respectively).

3.2. Molecular forms and degree of activation of MMP-8 and MMP-9 (III, IV)

Western immunoblotting analysis revealed both isoforms of MMP-8 at approximately 70 to 80 kDa representing activated and latent forms of neutrophil (PMN)-type MMP-8 and two species at approximately 45-50 to 55-60 kDa representing activated and latent forms of non-PMN-type MMP-8 derived from the mesenchymal cells in patient samples. Mainly the mesenchymal-type MMP-8 was converted to active forms except in six patients, who had also detectable immunoreactivity of active PMN-type MMP-8. There was no difference in the degree of MMP-8 activation between the eosinophil-positive and eosinophil-negative patient. In re-operated patients the proportion of latent mesenchymal MMP-8 isoform, but not active mesenchymal isoform, was significantly lower than in those patients who were not re-operated (median 2.0%, IQR 0-4.1% vs. median 8.7%, IQR 6.2-9.9%, $P=0.02$). Also higher levels of >90 kDa high molecular size MMP-8 species were detected in samples from CRSwNP patients in relation to controls. A latent form of PMN-type MMP-8 was major MMP-8 species observed in NALs from controls.

Gelatin zymography revealed three forms of MMP-9 at approximately 120 kDa, 92 kDa and 82 kDa representing MMP-9-NGAL complex and latent and activated forms of MMP-9, respectively. All MMP-9 forms were detected in both patient groups and controls. In controls these forms were seen in equal proportions (median 33.0%, 33.7%, and 32.4%), whereas in patients the level of latent form was increased and active form decreased in relation to controls (median all patients 33.7%, 50.2% and 14.1%). There was no significant difference in the distribution of MMP-9 forms between the eosinophil-positive patients and eosinophil-negative patients, neither between the re-operated patients and those who were not.

3.3. Correlation between MMP-8 and cytokines (III)

The mesenchymal-type MMP-8 isoform but not PMN-type MMP-8 isoform was converted to active form as the IL-8 concentration increased in CRSwNP patients. A significant correlation ($r = 0.630$, $P<0.05$) between IL-8 and the proportion of the activated form of mesenchymal MMP-8 immunoreactivity was observed. The other

studied parameters (IL-8 or TNF-α vs. MMP-8 concentration; IL-8 or TNF-α vs. PMN-type MMP-8 immunoreactivity; TNF-α vs. the proportion of the activated form of mesenchymal MMP-8 immunoreactivity) did not show a significant correlation.

3.4. Correlation between MMPs and TIMP-1 (IV)

A significant correlation (r = 0.560, $P<0.01$) between MMP-8 and MMP-9 was observed. The other studied parameters (TIMP-1 vs. MMP-7, MMP-8 or MMP-9; MMP-7 vs. MMP-8 or MMP-9) did not show a significant correlation.

DISCUSSION

1. Patient characteristics

Patients were representative of typical CRSwNP patients. Fifty % reported allergic rhinitis, 57% had asthma and 29% of the asthmatic patients had ASA intolerance, which concurs with previous reports.[122,272,282,283,294] In the general population in Finland the incidence of allergic rhinitis is approximately 15-20% and the incidence of asthma is 2-6%.[120,133,263] Also the incidence of ASA intolerance among asthmatic CRSwNP patients was markedly higher than the reported incidence of approximately 9% in asthmatic patients in Finland.[133] In this study tissue eosinophilia was found in 19 patients (63%), of whom 13 subjects had also asthma and four subjects had ASA intolerance, whereas in the literature 80-90% of CRSwNP patients are described to have eosinophilic inflammation.[142] Secretion eosinophilia was found only in eight patients. Eosinophil staining was done on the NAL sample, and it is possible that NAL fluid does not contain a sufficient concentration of cells and mucus from sinus cavity to detect secretion eosinophilia. In the present study three patients had specific IgG subclass deficiency and one patient had both IgG subclass and IgM deficiencies. In several studies a high incidence of immunodeficiency among CRS patients has been demonstrated, with the IgG subclass deficiency being the most common immune defect in up to over 30% of CRS patients.[55,194,265] Our results suggest also that the polypoid form of CRS is associated with immunodeficiency. However, the small number of patients and patient selection have an influence on the results, as well as the fact that the patients entering this study were not tested systemically for immune dysfunction.

CRSwNP is known to have a high frequency of recurrence: 63% of the patients in the present study had been operated on earlier and similar numbers have also been described in literature.[91,122,158,281] In a long-term follow up study of nasal polyps the ASA intolerant patients had the highest frequency of recurrence and they usually needed re-operation and medication more often than patients with atopic allergy or intrinsic allergy-like disease.[318] The high recurrence frequency in ASA intolerant patients was also seen in the present study. All the patients with ASA intolerance had been operated on prevuously and four of these five patients had had several operations. Seventy-five % of asthmatic patients without ASA intolerance had had a previous operation compared to 54% of non-asthmatic patients. Moreover, in the three year period after the initial operation, nine out of 24 patients enrolled to the study IV (38%) were re-operated. Of re-operated patients, eight subjects had tissue eosinophilia, which is usually associated with poorer prognosis in CRSwNP.[99] Moreover, also seven re-operated patients were asthmatic. The number of patients is too small to make further conclusions but CRSwNS associated with asthma and

no ASA intolerance could have a poorer prognosis than CRSwNP in non-asthmatic patients. Every patient with immunodeficiency had been operated on earlier and three of these patients were also re-operated during follow-up period. Thus, the risk for recurrent operations appears to be associated with tissue eosinophilia, asthma, ASA intolerance and immunodeficiency. On the other hand, tissue eosinophilia was strongly associated with asthma and ASA intolerance, and so this may reflect a common pathophysiologic mechanism in CRSwNP patients with systemic and more active disease.

2. Microbiology of chronic rhinosinusitis with nasal polyposis

2.1. Bacteria

The bacteria culture results were similar to those of previous studies.[38,122,147,264,326] *Staphylococcus aureus* and coagulase-negative Staphylococci were the two most common isolates in both the control group and the patient group. *Pseudomonas*, which is reported to be associated with CRS, was not recovered from any patient or control sample.[209]. However, in the patient group other bacterial species, which are rarely discovered from healthy noses or acute community acquired rhinosinusitis, including *Klebsiella*, *Escherichia coli* and *Proteus*, were isolated. The polymicrobial aetiology of CRS, consisting particularly of aerobic and anaerobic beta-lactamase-producing bacteria, is highlighted in some studies.[27,41] Beta-lactamase-producing bacteria were found in the present study, but their pathogenity in CRS is unknown. The same bacteria are also the main components of the normal nasal flora.[147,264] However, it is hypothesized that the normal nasal flora may be the normal flora solely under healthy conditions.[27] The only anaerobic bacteria isolated from three patients' samples and one control sample taken in September was *Propionibacterium acnes*. Aral and colleaques found anaerobes in 14.2% of maxillary sinus aspirates taken during ESS whereas only aerobes grew in the ethmoid sinus samples and nose swap samples from the same patients.[7] Samples taken "deeper" inside the nose could favour anaerobes, as well as the changes in the sinus microenvironment during chronic rhinosinusitis, such as impaired ventilation and reduced oxygen levels.[40] On the other hand anaerobic bacteria, especially *Propionibacterium* species are found in up to 100% of nasal cavities of healthy subjects.[147]

Bacteria may be implicated in CRS by other mechanisms than those causing chronic infection. Several bacterial species implicated in chronic rhinosinusitis are potent inducers of IgE pointing to a possible allergic mechanism.[27] One of the most likely candidates in CRSwNP is superantigen producing strains of *Staphylococcus*

aureus, which are supposed to cause eosinophilic inflammation of the sinus mucosa in genetically susceptible hosts.[12,266] Specific IgE to *Staphylococcus aureus* enterotoxins A and B has been demonstrated in 50% of altogether 20 patients with eosinophilic nasal polyposis.[12] The presence of *Staphylococcus aureus* enterotoxin specific IgE associated with higher levels of total serum IgE, more severe local disease and increased incidence of systemic manifestations (asthma), suggesting that superantigens can impact on disease severity. In the present study, eight of ten patients with *Staphylococcus aureus* had been operated on at least once earlier, but the prevalence of *Staphylococcus aureus* was about the same in the control group. Longer courses of the disease and several operations could also have influenced the normal nasal flora, which could favour *Staphylococcus aureus*. Moreover, no classification of different types of *Staphylococcus aureus* was performed in the present study, making it impossible to estimate the superantigen theory in the persistence of disease.

2.2. Fungi

Seven patients were diagnosed to have possible fungal rhinosinusitis. The criteria of sinus mycetoma were met in three patients. In two of them there were fungal hyphae consistent with *Aspergillus* in biopsy and in one patient the fungal culture was positive for *Aspergillus fumigatus*. The remaining four patients were considered as having AFRS-like syndrome, because the diagnostic criteria in AFRS vary and the atopic status of patients was not analyzed. No invasive fungal infections were discovered. The fungi were present either in the direct microscopy of the sinus mucus or in the histological examination, representing thus a real fungal finding, not a contamination of a sample. Culture was positive in only two patients, who had also fungal hyphae present in the fungal staining of sinus mucus. This reflects the poor viability of the fungi and concurs with the estimation that only 20-40% of fungal infections are culture positive.[110] Based on this finding it can be concluded that fungal staining of mucus as well as histological samples stained with specific fungal staining are needed to detect fungi.

The occurrence of fungal rhinosinusitis in this study (23%) is high when compared to the estimated 5-10% prevalence in the literature.[110,190] However, an equal prevalence of fungal positive findings was found in a study of 117 immunocompetent patients having CRS together with a presence of granulomatous and friable material within the sinus in endoscopy.[317] In that study 25.7% of the surgical specimens were positive for fungi with *Aspergillus* species being the most prevalent fungal genera. The authors concluded that the results represent a fungal colonization of the paranasal sinuses instead of a mycosis. On the other hand, their results may indicate that when the typical findings are present, there is strong

possibility of fungal infection. A much higher incidence of fungal findings, exceeding over 90 %, in the adult CRS patients has been found in some other recent studies.[32,45,246] It is not known whether or not the fungi can exist in the sinuses without causing a disease. However, the absence of any difference between the patient and the control fungal culture results in the nasal swabs and NAL samples simply reflects the fungal colonization of the nose.[32,45,50,246] This has been demonstrated also in a study of 30 neonates, whose nasal mucus samples yielded positive fungal culture in 15% on the fourth day postpartum and the number increased to 94% in four months.[174] In the present study five of seven fungus positive patients (71%) were operated on earlier. That does not differ significantly from the average 63% of all patients in this study. In our view the presence of fungi does not explain the chronic course of CRSwNP. However, it should be kept on mind, that some patients with typical clinical picture of CRSwNP may actually have fungal rhinosinusitis, in which case early operative treatment is the treatment-of-choice.[82-84]

2.3. Seasonal variation in controls

The presence of two common airborne outdoor fungal species, namely *Cladosporium* and *Alternaria*, was clearly seen in the autumn samples, whereas every sample taken in January was negative for fungi. These results reflect the fungal spores in the patient's outdoor environment, as the same fungal species were also seen in high levels in the air at the same time. This theory is supported further by the finding that the most common airborne indoor fungal genera, i.e. *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria* and *Aureobacidium*, are also the most prevalent isolates in NAL samples from CRS patients and controls in several studies.[32,45,53,246] Moreover, *Cladosporium* spp., *Alternaria* spp. and *Aureobacidium* spp. showed a significant seasonal fluctuation with the maxima in late summer/early autumn.[45] In our study the similar seasonal variation was not seen in the patient samples. The patients' samples were collected directly from the sinuses, which probably explain the difference. The paranasal sinuses are not so easily accessible by fungal spores in the inhaled air, especially if the spores are big like those of *Alternaria* and *Cladosporium*. The results of Buzina and colleagues support this theory.[45] In their study 91.3% of 104 mucus samples obtained by NAL method from CRS patients yielded positive fungal culture compared to 84.0% of 106 samples obtained during ESS. However, the occurrence of some taxa, *Cladosporium* for example, was significantly higher in NAL samples (65.2% in controls and 42.3% in patients) than in samples collected from the sinuses during surgery (20.8%). The bacterial culture results in the controls show that the normal nasal bacterial flora varies according to time. More positive bacterial cultures grew in the autumn samples, but the bacterial species found did not differ much from the

winter results or the species isolated from the patient samples. It was mainly the increase in the presence of the coagulase negative staphylococci, which explains the increase in the number of positive bacterial cultures in autumn.

3. Impact of moisture exposure on chronic rhinosinusitis with nasal polyposis

The presence of moisture has become a popular explanation for different respiratory and general symptoms. This together with the need of patients to find a cause for illness might lead to an overestimation of the association between moisture problems and the symptoms. In the present study the patients had objective findings of CRSwNP, which is probably less sensitive to reporting bias compared to subjective estimation of symptoms. Moreover, patients reported mainly obvious water damage, visible mould or repairs done due to the moisture damage, which are clear signs, not only suspicion of moisture damage. An association between fungal rhinosinusitis or CRSwNP and exposure to dampness was not apparent in the present study. Altogether 46% of patients reported exposure to moisture at home or at work in this study. It is high compared to the 15-23% prevalence of damp/mouldy homes in two other Finnish questionnaire surveys.[161,241] The higher prevalence of reported moisture problems may be partly due to the fact that we estimated exposure to moisture both at home and at work. Moreover, the last three years instead of the last one year typically assessed in questionnaire surveys of this nature were evaluated in this study. However, the found prevalence is comparable to another Finnish study where a random sample of 310 houses was studied.[167] A moisture problem was observed by a surveyor in 52% of the houses, whereas a mould problem was reported by the occupants in 27%.

In this study three out of six patients with fungal rhinosinusitis reported moisture exposure, which is in accordance with the 46 % prevalence of moisture damage in patients altogether. The variants of fungal rhinosinusitis were distributed evenly: one mycetoma patient and two with an AFRS-like syndrome in both exposed and non-exposed patients. There was a marginally higher occurrence of previous nasal or sinus operations among the patients exposed to moisture (77% vs. 60%), but repeated operations were reported equally suggesting that there was not a significant difference in the persistence of the disease between the groups. Tissue invasion by indoor moulds is evidently rare except in persons who are severely immunocompromised.[128,316] However, there may be alternative mechanisms to explain how moisture, mould growth or possibly other factors co-existing in a damp environment might cause chronic mucosal irritation and/or impaired mucociliary clearance leading to the development of CRS. For example, up-regulation of inflammatory mediators due to the environmental exposure may also

alter the inflammatory cascade in the nasal and sinus mucosa. For example, nitric oxide favours the expression of Th2 cells by being less inhibitory to Th2 cells than Th1 cells.[155] The elevated levels of nitric oxide have been detected in NALs from the staff working in a mould-contaminated school, whereas there is evidence of Th2 cell involvement in allergic as well as non-allergic rhinosinusitis.[16,125,137,249]

The prevalence of tissue eosinophilia was higher in exposed group (75% vs. 53%). The increased concentrations of ECP, a marker of the eosinophil activation, have been detected in NAL samples from people working in damp office building and from subjects exposed to formaldehyde, nitrogen dioxide, and *Aspergillus* spp.[218,329] No mucosal biopsies from the nose or the sinuses were examined in these studies, but these findings are still interesting, because the eosinophilic inflammation is considered as a key event in the pathogenesis of CRSwNP.[13,99,172] Moreover, the presence of tissue eosinophilia in CRS is associated with more severe disease and an increased need for repeated operations.[99] These results from previous studies may provide a possible link between environmental exposure and chronic inflammation of the nasal mucosa. Thus, some connection between environmental exposure and CRSwNP may exist, although we did not find an association between them.

4. MMPs and TIMP-1 in chronic rhinosinusitis with nasal polyposis

There are only very limited data concerning MMPs in CRS, but MMPs have been studied extensively in lower airway diseases. The characteristics of inflammation are rather similar in CRSwNP and asthma, and thus they may represent, at least in part, a different clinical picture of the same pathophysiological process.[30,196,245] Thus, the results from our studies are discussed in respect to previous findings in CRS as well as in lower airway physiology and pathology, especially in relation to findings in asthma. Along with the expression of MMPs the MMP/TIMP-1 molar ratio is an important aspect when estimating MMP functions and roles in disease pathogenesis, as the balance between them is though to be critical factor in regulating the breakdown of connective tissues and the immune reactions by MMPs.

In this study the levels of MMP-7 in eosinophil-negative CRSwNP patients were statistically significantly elevated in relation to controls, but comparable with eosinophil-positive patients. However, the MMP-7 concentrations were overall very low (IQR 0.008-0.015 pmol/mL in eosinophil-negative patients) and did not exceed TIMP-1 concentrations and are thus probably of minor clinical and pathophysiological importance. MMP-7 is one of the few MMPs which are

constitutively expressed in non-injured, normal epithelium, where it is considered to contribute to tissue homeostasis and innate immunity.[233,341] The elevated MMP-7 levels have been found also in CRSwNP.[336] In particular, the inflammatory cells stained positive for MMP-7, as well as for MMP-9 were found inside the pseudocysts, pointing to their possible degradative function in nasal polyposis pathogenesis.[336] In the present study MMP-7 was measured from sinus mucus/NAL samples, which could explain the difference from the previous results. The spatial distribution of MMP-7 is distinct in repair and inflammatory functions, as the wound induced MMP-7 is released basally towards the underlying matrix compared to its release to airway lumen away from matrix in other functions.[233] On the other hand, bacterial exposure in mucosal epithelial tissues induces 25-50 fold increase in expression of MMP-7.[187] Since no such increase was seen in the present study, it seemingly provides more evidence of non-infectious etiology of CRSwNP.

Concurrent with previous reports, we observed a significant increase in MMP-9 concentration in sinus mucus from CRSwNP patients. Moreover, the patients without tissue eosinophilia in sinus mucosal biopsy had significantly elevated levels of MMP-9 when compared to eosinophil-positive patients and controls, whereas in eosinophil-positive patients these parameters were at the same level compared to controls. A similar distribution was seen in the MMP-9/TIMP-1 molar ratio, with the median ratio in eosinophil-negative patients, eosinophil-positive patients and controls being 2.97, 0.38 and 0.22, respectively. There was no difference in the degree of activation of MMP-9 between patients groups. In the previous studies the elevated levels of MMP-9 and TIMP-1 has been found in CRSsNP, whereas in nasal polyposis MMP-9, but not TIMP-1, was up-regulated.[180,336] TIMP-1 is considered to have fibrogenic properties resulting from inhibition of MMP-9 and promotion of growth of fibroblasts and myofibroblasts seen also in nasal polyp tissue in contrast to normal nasal epithelium.[131,332] The TIMP-1/MMP-9 imbalance could thus lead to pathologic collagen deposition seen in CRS and asthma.[29,193,284,321] Unfortunately the MMP-9/TIMP-1 molar ratio has not been reported in the previous studies, but the up-regulation of TIMP-1 in non-polypoid form of CRS could explain the prominent fibrosis found in this subgroup, but not in CRSwNP.[207] On the other hand, depending from the MMP function, lack of inhibition of MMPs in nasal polyp tissue could lead to pseudocyst formation by tissue destruction.

These findings considering up-regulated MMP-9 expression in non-eosinophilic inflammation were unexpected, since MMP-9 has been implicated in eosinophil migration through basement membranes.[224] In addition, MMP-9 is produced by many cells that accumulate in allergic airway inflammation including eosinophils, and MMP-9 gene deletion is associated with impaired transmigration of dendritic

cells to the airway lumen and decreased peribronchial eosinophilic inflammation.[9,48,193,223,320] Tissue eosinophilia in CRS is associated with more active disease. In this respect, our results are in contrast to the previous findings revealing that MMP-9 expression in the nasal mucosa and its concentration in sinus fluid after sinus surgery are linked with poor healing estimated by endoscopy, although it did not correlate with edema, fibrosis, or inflammatory cells in tissue.[337] The samples in the present study were collected during operation and the prognosis was estimated as a need for re-operation, which may explain the difference. Probably the reason for seemingly incongruent results is alterations of MMP functions in different disease phases. In vitro studies in Ewing's sarcoma cell line have shown that enhanced MMP-9 expression results in decreased levels of surface E-cadherin by mechanism not related to decreased proteolysis of surface E-cadherin.[262] E-cadherin is a cell-surface glycoprotein involved in cell-to-cell adhesion and is responsible for the organization, maintenance, and morphogenesis of epithelial tissues. Thus, an increase in MMP-9 expression after operation in relation to poor outcome may reflect disturbances in tissue repair mechanisms, whereas preoperative/intraoperative up-regulation MMP-9 may result from several different reasons varying from tissue homeostasis to disease pathogenesis.

A significant increase in MMP-8 concentration was seen in a subgroup of CRSwNP patients entered in the study III in relation to healthy controls. Mainly the mesenchymal-type MMP-8 isoform (45-55 kDa) was converted to active forms except in two patients, who had detectable immunoreactivity of active 65-75 kDa PMN-type MMP-8, perhaps representing the acute exacerbation of CRSwNP. In controls instead 80 kDa latent form of PMN-type MMP-8 was major MMP-8 species observed in NALs. However, in study IV the CRSwNP patients altogether did not show enhanced MMP-8 expression relative to controls. The degree of MMP-8 activation was similar to that found in study III. When patients in the Study IV were divided to two subgroups according to tissue eosinophilia, a clear difference in proteolytic pattern was found. As in MMP-9 expression, also MMP-8 concentration was significantly higher in CRSwNP patients without tissue eosinophilia when compared to eosinophil-positive patients and controls. Again, there was no statistically significant difference between eosinophil-positive CRSwNP patients and controls, nor did the patient groups differ in the molecular forms and degree of activation of MMP-8.

MMP-8 has not previously been studied in CRS with or without nasal polyposis. MMP-8 is regarded as playing a central role at sites of matrix, especially type I collagen, degradation associated with inflammation.[127,228,252] Elevated levels of both PMN-type and mesenchymal-type MMP-8 isoforms has been found to be converted to active forms in BAL fluid from patients with bronchiectasis, a disease

characterized by irreversible tissue injury.[253] In asthmatic patients the secreted MMP-8 in BAL fluid was converted into active form in steroid naïve or uncontrolled severe disease but not in clinically stable disease, nor in healthy controls.[252] In that study MMP-8 immunoreactivity was detectable especially in the bronchial epithelial cells in the areas of injured airway epithelium. In asthmatic airways high levels of activated MMP-8 probably indicates more advanced destructive and irreversible tissue injury rather than inflammation itself. In this respect, it is possible that activation of mesenchymal-type MMP-8 in CRSwNP emphasizes repeatedly damaged mucosal epithelial lining as well. In addition, differences in both MMP-8 and MMP-9 expression between eosinophilic and non-eosinophilic CRSwNP suggest that different pathophysiologic mechanisms can be involved in these subgroups.

4.1. Activation of MMP-8 in correlation to increase in IL-8 concentration

Activation of mesenchymal-type MMP-8 was associated with elevated levels of IL-8 but not TNF-α. IL-8 is a potent chemoattractant for and inducer of neutrophil degranulation, but there is limited data that it can act as a chemoattractant for eosinophils in allergic persons.[257,335] The sinus mucosal expression of the IL-8 gene was increased in patients with chronic rhinosinusitis.[257] Moreover, the level of IL-8 gene expression correlated with the disease severity assessed by sinus CT scores and with symptom scores. Interestingly, the incidence of IL-8 gene expression did not vary with allergic or asthmatic status, the presence or absence of polyps, or the use of corticosteroids. Thus, it is not known if IL-8 expression is actually dysregulated in CRS or does it rather represent an innate immune response to sinus infection.[31]

IL-8 is known to stimulate MMP-8 expression and secretion.[314] A significant correlation ($r = 0.628$, $P<0.02$) between IL-8 and the proportion of the activated form of total mesenchymal MMP-8 immunoreactivity was observed in the present study. The regulation of MMPs is complex and occurs at different levels including gene transcription, mRNA stability, synthesis, secretion, proenzyme activation and inhibition by specific and non-specific factors. However, taking into consideration the close relationship between the inducer and target, IL-8 and MMP-8 may form a pivotal inductive cytokine-proteinase cascade in the pathogenesis of CRSwNP. This finding is different from the earlier theory that eosinophil granule proteins mediate tissue damage in chronic rhinosinusitis.[13,99,129] MMP-8 is not implicated in development of eosinophilic inflammation, a major histological hallmark of CRSwNP. An IL-8/MMP-8 relationship may, at least in part, explain the mechanism of macrolide antibiotics, which has been shown to be as effective as prednisolone in chronic rhinosinusitis in long-term, low-dose administration.[331]

Macrolide antibiotics can produce a significant reduction in IL-8 production, which subsequently in turn may lead to reduced MMP-8 levels and activation.

In study III the high prevalence of asthma among the CRS patients was first suspected to explain the up-regulation of MMP-8 in the patient group. In contrast to that what was expected, the level of MMP-8 expression was lower in asthmatic than non-asthmatic patients, although this difference was not statistically significant. However, considering the results from study IV, this difference was probably due to different proteolytic pattern in eosinophilic and non-eosinophilic inflammation, since eosinophilia associated strongly with asthma. Therefore, the possible inductive IL-8-MMP-8 cascade in CRSwNP in relation to tissue eosinophilia needs to be studied further in larger patient population and in different CRS subgroups.

4.2. The protective role of MMP-8 and MMP-9 in chronic rhinosinusitis with nasal polyposis

We demonstrated enhanced in vivo MMP-8 and MMP-9 expression together with increased MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios in non-eosinophilic chronic rhinosinusitis with nasal polyposis, whereas in eosinophil-positive patients these parameters were at the same level compared to controls. Also in patients with more active disease, estimated as a need for re-operation during the three-year-period after initial operation, the levels of MMP-8, MMP-9 and their molar ratios to TIMP-1 were significantly lower than in patients who were not re-operated. Enhanced and persisting allergen-induced airway inflammation involving increased number of eosinophils in MMP-9 deficiency in mice may indicate defensive reaction of MMP-9 in lung injury following allergen challenge and especially the importance of MMP-9 in its resolution.[195] This is in accordance with the known function of gelatinases in the transepithelial migration of inflammatory cells, including eosinophils, in the bronchial mucosa.[67] MMP-2 and MMP-9 regulate the formation of transepithelial C-C chemokine gradients, which conduct the extravasated inflammatory cells through the pulmonary interstitium and airway epithelium to enter the airway lumen, where they are cleared. Lack of MMP-2, affecting only eotaxin (CCL11), and MMP-9, affecting eotaxin, CCL7, and CCL17, disrupts the normal cell trafficking into the airway lumen and favour their accumulation in parenchyma. Thus, by generating several transepithelial chemokine gradients, MMP-9 is essentially implicated in the resolution of allergic/eosinophilic inflammation. It most likely serves the same function in CRSwNP, as also eotaxin is implicated in the accumulation of eosinophils in this disease.[11,276]

As in MMP-9, the lack of MMP-8 up-regulation associated with poorer prognostic factors in eosinophil-positive patients may indicate defensive role of MMP-8 in CRSwNP. Furthermore, in non-eosinophilic CRSwNP patients the MMP-8/TIMP-1 molar ratio was markedly higher than the MMP-9/TIMP-1 ratio. This difference may implicate also more important function of MMP-8 in inflammatory control. The functions of MMP-8 in inflammatory disorders are not well established, making it impossible to evaluate the possible reciprocal relationship of MMP-8 and MMP-9. However, based on previous result, synergic function seems more plausible. MMP-8 and MMP-9 deficiencies are both associated with reduced inflammatory cell apoptosis and prolonged inflammatory response in allergen-induced airway inflammation.[111,195] Moreover, similar inflammatory features consistent with characteristic inflammatory features in CRSwNP, namely enhanced levels of Th2 cytokines and eosinophils, have been found both MMP-8 and MMP-9 knock-out mice in allergen-induced airway inflammation, suggesting synergic function of MMP-8 and MMP-9 in the control of Th2 inflammation.[111,195] The strong positive correlation ($r = 0.560$, $P<0.01$) between MMP-8 and MMP-9 in patients in the present study provide further evidence of their synergic functions in the airway inflammation. In addition, MMP-9 is known to enhance IL-8 activity, which in turn may result in the activation of MMP-8, as seen in study III.[313]

The possible anti-inflammatory function of MMP-8 and MMP-9 was further confirmed, when patients who were re-operated on due to CRSwNP during the three-year period after initial operation were compared to not re-operated patients. Nine patients were re-operated on, of whom eight had tissue eosinophilia, although there was no statistically significant difference in the risk for re-operations between the eosinophilic and non-eosinophilic CRSwNP patients. However, significantly elevated levels of MMP-8 and MMP-9 concentrations and MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were found in patients who did not need re-operation in relation to re-operated patients. The lower MMP levels in eosinophilic CRSwNP in general do not seem to fully explain the difference between these patient groups, since partly similar difference was seen when comparing only eosinophilic CRSwNP patients. Also, in this subgroup the re-operated patients, in relation to not re-operated subjects, had lower MMP-8 and MMP-9 concentrations along with their molar ratios to TIMP, but only MMP-9 values reached statistical significance. Thus, it seems that up-regulation of MMPs is needed in reaction to different stimuli including perhaps also inflammation or infection in order to prevent prolonged injury to the affected tissues.

CONCLUSIONS

I. Microbiological findings do not seem to explain the chronic course of CRSwNP. The occurrence of fungal rhinosinusitis in CRSwNP patients exceeded the estimated 5-10% prevalence in rhinosinusitis in general, implicating association of this CRS subgroup with non-invasive fungal infections. The risk for recurrent operations for CRSwNP was associated with tissue eosinophilia, asthma, ASA intolerance and immunodeficiency, which may present malfunctions in systemic immunologic mechanisms in the pathophysiology of CRSwNP.

II. CRSwNP and fungal rhinosinusitis were not associated with exposure to moisture damage at home or in the workplace. Environmental exposure reflects to the fungal findings in the nasal cavity. This should be taken into account, especially when the NAL samples are used for microbiological studies.

III. MMP-8 expression was upregulated in CRSwNP patients. In particular, the mesenchymal-type MMP-8, but not PMN-type MMP-8, was converted to active form as the IL-8 concentration increased. TNF-α did not show a correlation with MMP-8. The observed IL-8/MMP-8 relationship may form an inductive cytokine-proteinase cascade in CRSwNP pathogenesis and may provide a target for novel anti-cytokine or anti-collagenase therapies.

IV. The proteolytic spectrum was different in eosinophilic and non-eosinophilic CRSwNP with the up-regulation of MMP-8 and MMP-9, as well as their molar ratio to TIMP-1, but not MMP-7, in non-eosinophilic form. This difference may reflect differences in pathogenesis in these subgroups and suggest a MMP-dependent mechanism in eosinophil-accumulation in CRSwNP. Eosinophilia in CRS is associated with more extensive disease and increased need for repeated operations, thus the lack of MMP up-regulation in eosinophilic CRSwNP in reaction to inflammatory stimuli may also indicate synergic protective/anti-inflammatory functions of MMP-8 and MMP-9 in CRSwNP. Similar difference was observed when re-operated patients were compared to patients who did not need re-operation. As MMP inhibitors are proposed to be of potential therapeutic value in chronic respiratory tract diseases, the better and more detailed understanding of MMP functions in inflammatory conditions is needed.

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